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The Application of Bacteriophage for the Elimination of Pathogenic Bacteria in Compost

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THE APPLICATION OF BACTERIOPHAGE FOR THE ELIMINATION OF
PATHOGENIC BACTERIA IN COMPOST

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Microbiology

by
Spencer D. Heringa
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Accepted by:
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ABSTRACT

The demand for organically grown produce is rapidly rising in the United States, resulting in a resurgence of natural fertilizers, such as animal waste, being used in agriculture. Currently, there are guidelines set by the USDA for the proper processing of fecal materials through composting; however food-borne illnesses associated with fresh-produce have become more and more common. Research is being conducted toward developing more effective treatments of fecal waste to reduce the survival of harmful bacteria. One possible form of treatment is through the use of bacteriophages that are able to “seek and destroy” specific bacteria. The objectives of this study were to: 1) isolate and characterize strains of bacteriophage capable of lysing *Salmonella* spp. and *Escherichia coli* O157:H7, 2) to develop and optimize a cocktail of these bacteriophages, and 3) to reduce pathogens in compost by administering bacteriophage to autoclaved and non-autoclaved composts inoculated with either *Salmonella* or *E. coli* O157:H7.

In the characterization and isolation of bacteriophage study, thirty-four *Salmonella*-specific and forty-two *E. coli* O157:H7-specific bacteriophages were isolated from raw sewage using an enrichment method. Ten phage strains for each pathogen were selected for use in optimization studies in liquid media. Phages exhibited little to no effectiveness in 0.85% saline or SM buffer, but were highly active in tryptic soy broth (TSB). Electron microscopy and restriction digest analysis were used to characterize the selected phages. A five-phage cocktail was developed and tested against five strains of *Salmonella enterica* encompassing four serovars: Typhimurium, Poona, Newport and

Enteritidis. The cocktail was effective at preventing the growth of *Salmonella* Typhimurium 8243 indefinitely in TSB. Treatments of phage cocktail suppressed the growth of other strains initially, but had little effect on preventing the development of phage resistance.

For the compost study, both autoclaved and non-autoclaved dairy compost was inoculated with 10^5 CFU/g of either *Salmonella* Typhimurium or *E. coli* O157:H7 and held for 24 h followed by phage treatment. Reductions of 2.34, 2.41 and 2.56 logs were observed in non-autoclaved compost inoculated with *Salmonella* Typhimurium 8243 at phage multiplicity of infections (MOIs) of 1, 10 and 50, respectively. An identical study using *E. coli* O157:H7 strain 0923-21 had reductions of 0.17, 1.21 and 1.5 logs with MOIs of 1, 10 and 50, respectively.

The impact of water activity in compost on pathogen reductions by phages was further studied. Compost prepared at water activity levels of 0.9, 0.95 and 0.98 yielded no reductions as a result of phage treatment in any of the trials. Non-autoclaved compost inoculated with *Salmonella* had reductions of 2 to 3 logs when the water activity was close to 1. Non-autoclaved compost inoculated with *E. coli* O157:H7 had a one log decrease after phage treatment. Only slight reductions in *E. coli* O157:H7 and *Salmonella* were detected in the autoclaved compost ($a_w=0.999$) as a result of 2 to 3 logs of bacterial growth prior to phage treatment.

The results indicate that specific bacteriophage cocktails are effective at reducing pathogen populations in compost under certain conditions, such as an appropriate ratio of phage to bacteria and high water activity. This suggests that phage treatment may be

useful as a supplement to composting for the further elimination of pathogenic bacteria in animal waste.

DEDICATION

I would like to dedicate this work to my parents, Ruth and Don Heringa, without whose support and encouragement this would not have been possible.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

In the United States, over 910 million tons of agricultural waste is produced annually (Environmental Defense, 1999). Several techniques for the removal of this biomass have been utilized, such as incineration and dumping, but by far the oldest and most common one is simply to spread waste material over agricultural land (Castro del Campo, 2007, Jones and Martin, 2003). The banning of waste dumping by the EPA in 1991 and the relatively high cost of incineration has left few other alternatives. The use of land application is favored especially because of the abundance of nutrients found in agricultural waste in addition to its relatively low cost. The spreading of unprocessed fecal material directly onto crops is often employed by other countries but has led to significant outbreaks of food borne illness (Ibenyassine et.al., 2007; Jerngklinchan et. al., 1993; Ponka et. al., 1995). Gerba and Smith (2005) state that untreated wastes can carry as many as 150 different enteric pathogens, highlighting the need for some form of treatment prior to land application.

As a result of the growth of the agricultural industry in the United States along with an increase in the occurrence of antibiotic resistant pathogens, the EPA has set requirements for the proper processing of biosolids prior to land spreading (EPA, 1999). Since it has been well established that many human pathogens such as *Salmonella* spp. naturally reside in the guts of livestock, composting has been adopted as an effective way

to create a stable, pathogen-free material for land application (MØlbak et. al., 2005). The EPA standards that have been set for composting should, under ideal conditions, eliminate the presence of all harmful enteric organisms (Misra et. al, 2003). The fact remains however that the number of outbreaks in fresh produce is now becoming more prevalent than those associated with meats. It is essential that as the country focuses on “health awareness”, pointing more towards the consumption of fresh vegetables, that steps be taken to ensure food safety. The occurrence of recent outbreaks provides evidence toward the possibility that some contaminating organisms may indeed persist until land application and beyond (Mahon et. al., 1997; Campbell et. al., 2001; Mohle-Boetani et. al., 2001). Due to the relatively low infectious dose of many pathogens like *Salmonella* and *Escherichia coli* O157:H7, the presence of any detectable organisms presents a threat to the public (Tarr et. al., 1994).

The composting process can eliminate pathogens via temperature increase, dessication, ammonia concentration and competitive exclusion (Shepherd et. al., 2007). One reason for pathogen persistence in compost may be the presence of cold spots or unprocessed regions due to improper turning of the compost pile (Soares et. al, 1995). This is especially critical on the surfaces of the compost heaps which are considerably cooler than the center. Since the composting process itself is designed to eliminate pathogenic microorganisms, little research has focused on additional treatments of the composting materials to safeguard against persisting organisms. Some studies have tested the effectiveness of ammonia and carbonate, urease inhibitors and thymol to reduce pathogen presence, but all conclude that further treatments would be necessary

(Park et. al., 2003, Diez-Gonzales, et. al., 2000, Wells, et. al., 2008). Other studies have tried to use irradiation which is an effective but less realistic way of treating large amounts of manure and other wastes (Monteith et. al., 1986). Furthermore, compost that has the majority of its background microflora inactivated may be more susceptible to re-population by pathogenic microorganisms from various sources such as animal droppings or cross contamination (Hussong et. al., 1985).

Bacteriophages (phages), which are viruses for bacteria, were discovered nearly a century ago but have become a topic of recent attention due to the increasing need for effective ways to eliminate pathogenic microorganisms. While the presence of these pathogens is a threat to the agricultural and produce industries, the dissemination of antibiotic resistance rapidly through bacterial populations causes additional alarm. Bacteriophages have a unique ability to find and infect specific bacterial hosts, in the process generating dozens to hundreds more infectious virions. This review focuses on the potential for bacteriophage application on the surfaces of compost to eliminate persisting *Salmonella* and *E. coli* O157:H7 during composting. To my knowledge no research has been performed to study the effect of bacteriophage application into compost to reduce pathogen prevalence.

***Salmonella* spp.**

Salmonella spp. are Gram-negative microorganisms that have a rod shaped appearance and can grow facultatively. Although commonly associated with enteric fever as a result of infection by *Salmonella enterica* subsp. *enterica* serovar Typhi

(*Salmonella* Typhi), the organisms most responsible for food-borne illness fall under the other *Salmonella enterica* serovars. There are well over 2,000 different serotypes of non-Typhi *Salmonella* species, among which is the recently discovered *Salmonella* Typhimurium DT104, which harbors multiple antibiotic resistances (Cormican et. al., 2002). While generally considered to be a pathogen associated with poultry (O’Flynn, 2006), *Salmonella* are also the major enteropathogen contaminating fresh produce (Aruscavage et. al., 2006; Lapidot et. al., 2005) and have been associated with outbreaks occurring in melons, alfalfa sprouts, black pepper, peanuts, and tomatoes (Brockmann, S., 2001; Emberland, et. al., 2007; Gustavsen et. al., 1984; Little, C., 2001; Greene et. al., 2008). *Salmonella enterica* serotypes present a serious health problem, being responsible for more than 40 thousand illnesses annually in the United States, with estimates of more than thirty times unreported, as well as 582 deaths (Mead, 1999). Costs associated with medical expenses and loss of productivity is estimated in the several billions of dollars each year (Voetsch, 2004). Worldwide, salmonellosis affects as many as 3 billion people resulting in millions of deaths (Wollin, 2007).

Infection occurs by the organism’s ability to survive in the phagosomes of macrophages, resulting in symptoms such as diarrhea, vomiting and abdominal pain (Rathman et. al., 1997). Transmitted mainly through the fecal-oral route, some serotypes may require the ingestion of as few as 10 cells to achieve infection, depending on the immune status of the host (MØlbak et. al., 2006). Virulence, which is most likely associated with the production of an enterotoxin, is compounded by the fact that many strains harbor a 43-kb pathogenicity island containing genes that confer antibiotic

resistance for a wide spectrum of antimicrobial agents (Cogan et. al., 2003).

Additionally, the presence of a plasmid bound integron has been found to be responsible for antibiotic resistance (Boyd et. al., 2002).

Of particular concern in regards to produce safety is the potential for both the persistence of pathogens or formation of biofilms on the surface of produce, as well as the internalization into the stem or root system through contaminated water or soils. Greene et al (2008) traced a series of *Salmonella* Newport infections that had occurred three years in a row to a farm in Virginia linked to contaminated tomatoes. Multiple *Salmonella* serotypes were detected in a nearby water source, with evidence to suggest that a rain event could easily contaminate the pond used for irrigating the tomato field. Contamination of the stem or flower has the potential to result in contaminated fruit (Guo et. al., 2001). A study by Penteado et. al. (2004) found that 83% of mangos that were placed in contact with water contaminated with *Salmonella* Enteritidis were positive for internalization of the stem, 19% into the blossom and 9% into the fruit segments. The surfaces of produce are also subject to the formation of biofilms which are markedly more resistant to many forms of treatment. A study by Lapidot et. al. (2005) inoculated parsley with *Salmonella* Typhimurium and discovered that although pathogen reductions were observed after treatment with a chlorine solution, the organism was able to recover and propagate. These and other studies demonstrate that once pathogenic microorganisms gain access to fruits and vegetables, they are extremely difficult to be eradicated. Therefore it is essential that harmful bacteria be eliminated before they are able to achieve contact with produce.

***E. coli* O157:H7**

E. coli O157:H7, a Gram-negative bacillus, was first identified as a clinical isolate in 1982, associated with contaminated ground beef. Infection is characterized by gastroenteritis and bloody diarrhea with the absence of fever in most cases. It was later linked to hemolytic uremic syndrome (HUS), which is a leading cause of kidney failure in young children (Besser et. al., 1999) and is now considered to be responsible for 85-90% of all cases of HUS (Armstrong et. al., 1996). *E. coli* O157:H7 is responsible for an estimated 70,000 cases and 60 deaths every year in the U.S. (Mead, 1999). There are currently six classes of diarrheagenic *E. coli* strains with *E. coli* O157:H7 falling under the classification of enterohemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998).

A review by Armstrong et. al. (1996) described three of the main generalizations of *E. coli* O157:H7. The first of which is its ability to be isolated from both healthy and ill animals. Cray and Moon (1995) performed an experiment inoculating both adult cattle and pre-weaned calves with *E. coli* O157:H7. They discovered that both groups shed *E. coli* O157:H7 for up to 14 and 20 weeks, respectively, in both healthy animals and those exhibiting diarrhea. The second generalization is the wide geographic region in which *E. coli* O157:H7 can be detected. A study by Zhao et. al. (1995) sampled multiple feedlots from 14 different states and isolated *E. coli* O157:H7 from as far east as New York and as far west as California. The third generalization is that the pathogen is most prevalent in pre-weaning calves, which has been suggested to be a result of developing microbial populations of the rumen which may allow *E. coli* O157:H7 to have a dominant role for some time (Grauke et. al., 2002).

While *E. coli* O157:H7 is known to be carried by cattle (McGee et. al., 2001), it has also been found in sheep, pigs, wild birds, insects and slugs (Ogden et. al., 2005; Kaufmann et. al., 2006; Foster et. al., 2006; Moriya et. al., 1999; Sproston et. al., 2006). Such a wide variety of hosts presents difficult and unique challenges to the prevention of fecal contamination of food products, especially when considering that an infectious dose has been shown to be as low as 50 viable cells (Fratamico et. al., 2006). Since 1995 there have been 22 outbreaks in the United States associated with fresh lettuce or spinach contaminated by *E. coli* O157:H7 (Cooley et. al., 2008).

Although it has been generally assumed that enteric organisms do not survive well outside of the gut of animals, many studies have reported that *E. coli* O157:H7 strains can persist long enough to be transmitted into human food sources (Lau and Ingham, 2001; Mukherjee et. al., 2006; Gagliardi and Karns, 2000; Ingham et. al., 2005). Solomon et. al. (2002) found that *E. coli* O157:H7 from manure and contaminated irrigation water is capable of internalizing into the roots and traveling into the edible portions of lettuce. Another study found that *E. coli* was internalized in the roots of cabbage after a sewage spill occurred nearby (Wachtel et. al., 2002). It was not, however, detected in the edible portions of the plant. A similar study by Johannessen et. al. (2005) found that while *E. coli* O157:H7 persisted in soil for at least 8 weeks, no internalization into lettuce was detected. Gagliardi and Karns (2000) note that the survival of enteric organisms in the environment is dependent upon conditions within the soil itself, such as clay and sand contents. Regardless of whether or not *E. coli* O157:H7 is able to become internalized or

persist on the surface of different types of produce, the fact remains that its presence is still causing sicknesses and better methods to prevent its survival must be utilized.

Composting Practices

Composting is an aerobic, thermophilic process that involves the chemical and biological breakdown of biomaterials such as manure, sludges and carcasses, carried out primarily by various types of microorganisms. The result of the composting process is rich and stable humus-like material. Composting consists of three phases characterized by temperature as well as the major microbial populations (USEPA, 1999);

- 1) **Mesophilic phase** is the beginning of the composting process where temperatures slowly increase; enteric organisms are abundant at this stage.
- 2) **Thermophilic phase** can last from a few weeks to a few months with temperatures ranging between 50°C to 70°C and is driven mostly by thermophilic bacteria (Ishii et. al., 2000).
- 3) **Cooling/maturation phase** occurs below 40°C and results in finished compost with populations of mesophilic bacteria.

Several of the different composting methods used include windrows, aerated static piles and in-vessel composting. Windrows are commonly used on farms that produce large amounts of waste materials or in facilities that handle a great deal of materials. They are open air piles that are formed in long heaps and turned frequently for aeration. Static piles are often aerated through forced air flow from beneath the heap. Unlike the open air composting techniques, in-vessel composting is completely contained and is continuously monitored and controlled in terms of temperature, moisture and

oxygen content (Sherman et. al, 2005). In addition to having the optimal conditions for composting, the microbial flora is extremely important to the breakdown of biodegradable materials. Beffa et. al. (1996) highlights the importance of having mesophilic, thermotolerant and thermophilic bacteria, as well as actinomycetes and fungi. Not only do these organisms play a metabolic role in the compost but may be effective at eliminating pathogenic microorganisms through competition, modulation of the pH through respiration or direct antagonistic action.

When performed properly, complete inactivation of pathogenic bacteria is usually observed (Platz et. al, 1977; Ceustermans et. al, 2007). A study by Misra et. al. (2003) found that waste materials composted according to Environmental Protection Agency (EPA) standards can eliminate the presence of pathogenic bacteria, viruses, weed seeds and nematodes. The process however must be well maintained to certain levels of temperature, aeration and pH to ensure complete inactivation of the necessary microorganisms (Zhao et. al., 1995). In order to be considered safe for land application, the finished compost must be designated as a Class A, which according to the EPA indicates that pathogens such as *E. coli* O157:H7 and *Salmonella* spp. are undetectable in 25 g of compost (EPA, 1994). To ensure standardization, all aerated compost heaps must be maintained at 55°C or higher for 3 days for static heaps. The United States Department of Agriculture (USDA) also has set guideline similar to the EPA in requiring compost to be maintained at 55°C for a minimum of 3 days. Compost must also be passively or actively aerated and should cure in the mesophilic conditions for at least 45

days (USDA, 2002). Not only is this presumed to eliminate all potential pathogens but results in a soil amendment that is a source of slow released phosphorous and nitrogen.

Pathogen Survival during Composting

Although the majority of studies testing the persistence of pathogenic organisms in compost have found complete inactivation below detectable limits (Platz et. al., 1977; Ceustermans et. al., 2007; Pereira-Neto et. al., 1986; Wiley and Westerberg, 1969), it is important to note that most laboratory studies and many field studies do not accurately depict the variety of situations and formulas used in on-farm composting (Soares et. al., 1995; Hay et. al., 1996). Additionally, a few studies did discover that variability in the composting procedure resulted in varying degrees of pathogen survival. A study by Shepherd et. al. (2007) found that while *E. coli* O157:H7 inoculated at 7 logs per gram survived 14 days in the center of the heap, the organism was detected for 4 months on the surface. Furthermore, although the internal regions of the heaps reached 50°C for at least seven days, temperature stratification throughout the compost heaps was observed. Jones et. al. (1982) studied the persistence of *Salmonella* spp. in manure slurries and found that not only were the temperatures a factor, but the type of manure and specific serotypes of *Salmonella* were as well. Brinton and Droffner (1995) revealed that even materials composted at 62°C had allowed initial numbers of 7 logs of *Salmonella* and *E. coli* to be detected up to 59 days. When the study was taken to a laboratory setting, both *Salmonella* spp. and *E. coli* survived in composted food waste above 60°C for 5 and 9 days, respectively (Droffner and Brinton, 1995). A large scale survey of 72 composting

facilities by Hay et. al. (1996) found that *Salmonella* was present in biosolids in over half of the sites regardless of the fact that EPA requirements were met. If species of pathogenic organisms are indeed able to persist, even when EPA standardized composting techniques are met, improperly composted wastes present an even greater threat toward the integrity of agricultural products.

The importance of compost safety prior to land application has been well exhibited, and is especially evident with food outbreaks occurring in fresh produce, ranging from tomatoes (CDC, 2006) to sprouts (Feng, 1997) and many other types of fresh produce. Although the EPA criteria are well known, the compliance level is not well monitored. In many cases farmers do not have adequate storage space or just neglect to acknowledge this danger (Nicholson et. al., 2005). The potential therefore exists that high numbers of food-borne pathogens have direct interaction with produce that is often eaten raw. Lau and Ingham (2001) performed a study of *E. coli* survival when mixed with soil and found that the organism could be detected in high levels after a period of 19 weeks. Another study compared the survival of *Salmonella* Newport strains in dairy manure (You et. al., 2006). The original populations, verified by pulsed field gel electrophoresis (PFGE), were detected in non-sterilized and sterilized soil and manure mixtures after a period of 107 and 158 days, respectively.

Several studies have exhibited that *Salmonella* are able to persist in finished compost and manure-amended soils anywhere from three months to over a year respectively (Lemunier et. al., 2005, You et. al., 2006). A study by Feachem et al. (1983) discovered that *Salmonella* spp. inoculated directly into compost were killed at a much

higher rate than *Salmonella* confined within a glass container placed within the compost environment. However, a variety of composting substrates may contain materials capable of protecting such pathogenic organisms in a similar manner. Briancesco et. al. (2008) studied 20 different compost mixtures for the presence of *E. coli*, *Salmonella* and human parasites. *Salmonella* was present in compost at 2 logs per gram in compost composed of green waste, sewage and cellulosic discard. The presence of cellulosic materials may act as a protective agent for certain pathogens such as *Salmonella*.

There are multiple means by which pathogenic organisms persisting in compost may be able to gain access to fresh produce, both directly and indirectly. The direct use of improperly composted material as a fertilizer may result in produce contamination with pathogens via the surface or internalization routes. Wachtel et. al. (2002) and Solomon et. al. (2002) both showed that internalization can occur from sewage and contaminated irrigation waters, therefore the presence of contaminants in compost remains a threat. Indirectly, run-off from compost heaps has the potential to contaminate crops. Additionally, re-contamination and cross contamination can occur from the presence of feral animal droppings or the mixing of finished compost with fresh waste materials. Unfortunately many of the problems regarding contamination of compost materials come down to a personal responsibility that is often not recognized or realized, and thus contamination is allowed to occur.

Pathogen Regrowth in Compost

One major concern for a Class A compost is the possibility of pathogen re-growth, which has been seen to occur even after the population of the organism has dropped below detectable limits. A study by Russ and Yanko (1981) found that based on a carbon to nitrogen ratio greater than 15:1 and moisture content at least 20%, *Salmonella* were able to grow in raw sludge compost. They noted that in a less regulated composting system, in natural settings, the re-growth would likely be a great deal higher. Another study found that *Salmonella* were no longer detected in a windrow after 12 days of composting, but were then detected in the final cooling phase even though the temperature inside the compost pile had exceeded 55°C for as many as 16 days (Deportes et. al., 1998). Interestingly, Salter and Cuyler (2003) observed that the *Salmonella* population increased even during the thermophilic phase of compost performed according to EPA requirements. This presents a good example of the possible presence of undetected cooler regions in monitored compost. Shepherd et. al. (2007) reported the temperature is stratified inside the composting heaps in a field study, with surfaces allowing pathogen survival. While cooler regions may be one explanation, another possibility is the selection of heat resistant bacteria in the first phase of composting. Jiang et. al. (2002) found that the thermal tolerance of *E. coli* O157:H7, which was held in compost at 23°C for 24 h prior to heat treatment, was affected. Since it takes several days for the compost environment to reach high temperatures a selection of heat-adapted organisms could result in cell multiplication.

To further study the ability for re-growth after a re-population event, Lemunier et. al. (2005) seeded *E. coli* and *Salmonella* Enteritidis into green waste composted for four months and found significant increases of bacterial populations, up to 6 logs after one month and 5 logs remaining after 3 months. Pietronave et. al. (2004) studied the re-growth of *Salmonella* and enteropathogenic *E. coli* in finished organic waste compost with moisture contents of 10, 40 and 80% and found that significant re-growth only occurred in compost that was sterilized. This indicates the critical nature of having a background microbial population to aid in the prevention of pathogen dominance in the compost environment. Zaleski et. al. (2005) found that even *Salmonella* spp. that were reduced to meet EPA regulations, re-grew to above initial numbers upon exposure to more favorable environmental conditions such as a rain event.

The persistence of environmentally fit pathogens in addition to improper composting practices can result in organisms that have the potential to re-grow and be transferred to agricultural lands and crops. Further requirements by the EPA and even more strict guidelines may still be insufficient in eliminating all possibility of contamination. Biological control strategies, such as competitively exclusive microorganisms and bacteriophages have been proven effective against pathogens in live animals, meat products, water and against plant pathogens (Atterbury et. al., 2007; Bigwood et. al., 2008; McLaughlin et. al., 2008; Balogh et. al., 2006). Therefore, the use of bacteriophage at different stages of composting may prevent not only the re-growth of *Salmonella* or *E. coli* strains, but persistence throughout the process as well.

Bacteriophage

In 1915, Frederick Twort who was studying *Micrococcus*, noticed a strange clearing in certain colonies which were no longer culturable. Furthermore, when samples of cleared colonies were transferred to normal colonies they too exhibited clearing. At around the same time Felix d'Herelle found that liquid cultures of *Shigella* were cleared as a result of a similar phenomenon (Calendar, 2006). Regardless of who made the first bacteriophage discovery, it was d'Herelle who realized the immediate potential of this unknown substance to propagate on bacterial cells. In fact, bacteriophages were not readily accepted as viruses until the advent of the electron microscope in the 1940's allowed visual confirmation. This confirmation, however, was not needed for d'Herelle to begin bacteriophage trials, initially on chickens, to reduce outbreaks of *Salmonella* Gallinarium and soon after in human patients suffering from dysentery (Summers, 2001).

In the early 20th century, bacteriophage therapy spread quickly around the globe, with several large pharmaceutical companies in the United States offering phage cocktails. With the discovery of sulphonamides in the 1930's and other antimicrobials during World War II, phages were replaced in the West by these new, reliable and effective broad spectrum antimicrobials. Throughout this time however and still today, phage therapies are common in parts of Eastern Europe.

Bacteriophages are natural predators of bacteria and are estimated by many to be the most abundant organism on the planet; perhaps greater than 10^{31} mature virions (Atterbury, 2007; Mann, 2005). Phages are characterized based upon morphology as well as the composition of the nucleic acids which can be double stranded (ds) DNA,

single-stranded (ss) DNA, ds RNA or ss RNA. Many different phage morphologies exist, but by far the greatest percentage of over 5,000 characterized strains (over 96%) are the tailed phages (Ackermann, 2001). In general, phages consist of a protein capsid covering a single linear piece of nucleic acid. However, due to the level of diversity of phages, taxonomic classification has not been well established.

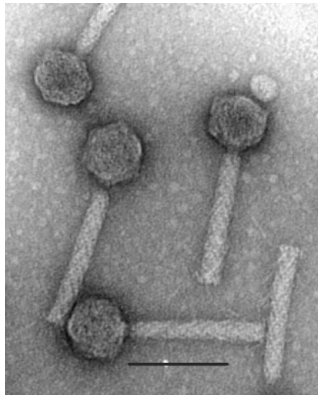
While relatively little is known about the population of phages in nature, there are several methods for the characterization of specific virions. The most common procedure is the use of electron microscopy, which allows for a visual confirmation and identification of specific families of phage (McLaughlin et. al., 2006). Other techniques such as pulsed field gel-electrophoresis (PFGE) and restriction-fragment length polymorphism (RFLP) analysis are employed to provide a more specific fingerprint of a particular phage strain (Raya et. al., 2006; Monod et. al., 1997). Perhaps the most important and informative procedure is the testing of a particular bacteriophage against a variety of hosts to determine a host range spectrum (Atterbury et. al., 2007). This can be used to distinguish different phages from one another.

For the purposes of this review, I will focus on the order *Caudelovirales* which contains the three main families of lytic tailed phages; *Myoviridae*, *Siphoviridae* and *Podoviridae*. Previous studies have already isolated and visualized phages from each of the three families that exhibit lysis against enteric pathogens including *Salmonella* spp. as well as *E. coli* O157:H7 (Whichard et. al., 2000; Sheng et. al., 2006).

The family *Myoviridae* (Fig. 1.1 and 1.2) contains such well studied phages as T4 and is characterized by the presence of an elongated head with a rigid tail, exhibiting

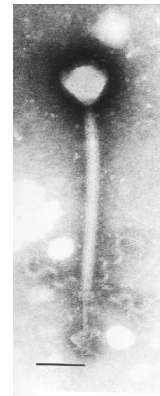
long, kinked tail fibers. Many of the genus in this family harbor modifications of the DNA bases, particularly 5-hydroxymethyl cytosine instead of cytosine or 5-hydroxymethyl uracil instead of thymine. A broad range of host strains are infected by phages within this family, both Gram-positive and negative, from a variety of environments (Calendar, 2006). The electron micrographs below provide an example of a phage infecting *Enterobacteria* and *Bacillus* (Fig. 1.1 and 1.2, respectively).

Figure 1.1



(Ackermann and DuBow, 1987)

Figure 1.2



The *Siphoviridae* family includes among others, the lysogenic phage lambda, and exhibits a long, non-contractile tail that is often quite flexible. So far none of the phages characterized in this family contain modified bases. Figures 1.3 and 1.4 demonstrate the flexible tail for phages infecting *Bacillus* and *Listeria* species, respectively.

Figure 1.3

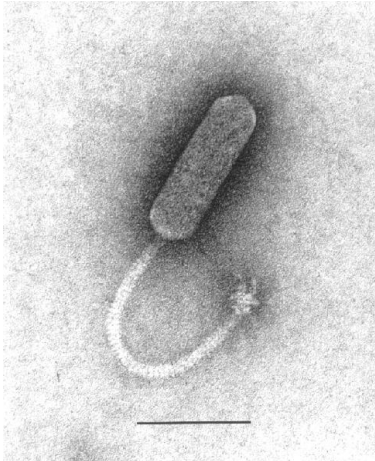
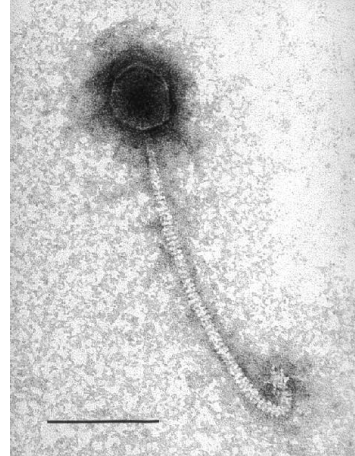


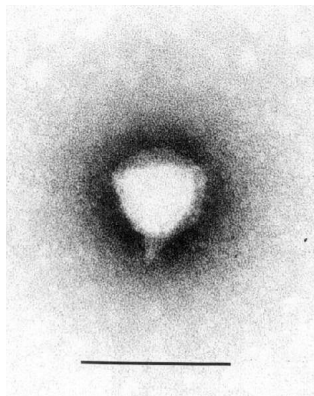
Figure 1.4



(Ackermann and DuBow, 1987)

Bacteriophages within the family *Podoviridae*, such as the lysogenic phage P22 discovered in *Salmonella* Typhimurium (Boyd et. al., 1951) can be identified as having a very short tail, often with tail spikes (Fig 1.5., host; *Brucella*). Similar to the *Siphoviridae* they lack DNA modifications and can be isolated over a broad host range.

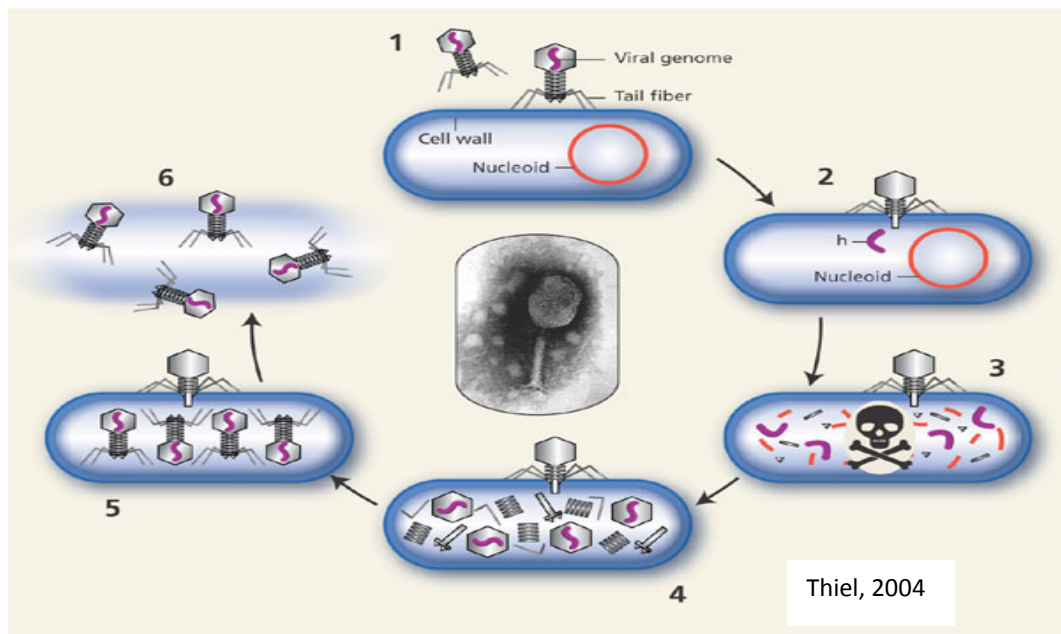
Figure 1.5



(Ackermann and DuBow, 1987)

Bacteriophage replication in lytic phage occurs upon random interaction between free phage and a potential host. Unlike the action of broad-spectrum antibiotics, bacteriophages adhere to specific receptors on the surface of bacteria, allowing them to target one strain or a number of strains of closely related bacteria (Sheng et. al, 2006). Upon adsorption of the phage to the cell surfaces, the nucleic acid is injected often along with initial proteins responsible for host metabolic take-over. Phages have many unique ways to take control of the host to produce virion bodies that are beyond the scope of this review. Once the cell's machinery is redirected towards the production of virions, dozens if not hundreds of mature phage are created, subsequently causing lysis of the host and distribution of the phage (Figure 1.6; Thiel, K., 2004).

Figure 1.6



Current Phage Applications

The potential for phage application has become a topic of extreme interest over the past several years, highlighted by several different reviews (Summers, 2001; Alisky, 1998; Barrow, 1998; Goodridge and Abedon, 2003; Greer, 2005; Hudson et. al., 2005). Most reviewers point to the current rise in antimicrobial resistance as the main reason for the need for new therapies. There are however many ways in which phage application are more beneficial than effective antibiotics including the ability to target specific pathogens, an increase in infective virions, and the lack of toxicity to both humans and animals. In fact, before the days of quality control, d'Herelle drank and injected phage solutions into his own and family member's bodies prior to attempts at use in others (d'Herelle, 1926).

Since bacteriophage has not been extensively studied in compost, it is important to take into account what studies have shown in other areas. Currently, a company called Intralytix, Inc., has been granted FDA approval of an anti-*Listeria* phage-based product for use as a food additive (Intralytix, 2006). The phage mixture LMP102 is composed of an equal combination of six bacteriophages specific to *Listeria monocytogenes*. Although no phage therapies have been approved for human use in the United States, the Republic of Georgia provides a commercially available wound-healing bandage preparation consisting of a mixture of lytic phages as well as an antibiotic in addition to readily available phage tablets for dysentery and other illnesses. A company out of Canada, Biophage Pharma, is looking to utilize bacteriophages towards cancer treatment as well as the elimination of *E. coli* and *Salmonella* Typhimurium in livestock and

carcasses. Other companies such as Exponential Biotherapies and Phage Therapeutic in the U.S., as well as GangaGen out of India, focus on the targeting of antibiotic resistant infections in humans (Thiel, 2004).

Phage Studies in Food Safety Application

Due to the increase in outbreaks related to fresh produce as well as contaminated meat researchers have started to focus on the possibilities of phage application in both pre- and post- harvest/slaughter situations (Table 1.1). Atterbury et. al. (2007) isolated over 200 bacteriophages and selected three based on the most effective killing over the broadest host range. These phages were fed to chickens after inoculation of the birds with *Salmonella*. The three separate phages resulted in log reductions of 4.2, 2.19 and no effect even though all three showed high effectiveness in laboratory experiments. This suggests the importance not only of phage effectiveness *in vitro* but also its ability to remain viable *in vivo*. Similar to the early experiments performed by d'Herelle to prevent *Salmonella* Gallinarium outbreaks in chickens, Toro et. al. (2005) used a cocktail of phage against *Salmonella* and achieved 6-fold reductions in the ceca. They too discovered that phage had a protective effect when it remained in the litter of birds as a result of consuming feed contaminated by fecal materials containing phage. An earlier experiment involving a pathogenic form of *E. coli* found that just by spraying phage on the litter of cows reduced the number of cases of diarrhea (Smith and Huggins, 1987). The use of an aerosol spray of bacteriophage over young broiler chickens inoculated with *E. coli* was attempted by Huff et. al. (2002), who found that while complete protection

was not provided, there was a significant decrease in bird mortalities among those treated with phage. Sheng et. al. (2006) applied bacteriophage specific to pathogenic *E. coli* O157:H7 directly to the recto-anal junction mucosa of cows resulting in 2-log reductions of the pathogen, however failed to eliminate the organism completely in shed feces. A similar study performed by Callaway et. al. (2008) inoculated a cocktail of bacteriophages in sheep to reduce levels of *E. coli* O157:H7. They found that a ratio of 1:1 was the most effective for reducing levels of pathogen in the gut by about 1 log per gram. None of the treatments however, reduced the level significantly in the rumen.

Whichard et. al. (2003) tested a known phage Felix 01 in reducing *Salmonella* Typhimurium DT104 levels on beef frankfurters as well as in liquid culture. They found that 300 CFU of *Salmonella* Typhimurium inoculated onto frankfurters were suppressed by as much as 2.1 and 1.8 log CFU/g. The effectiveness of phage on chicken skins was also tested against *Campylobacter* at multiple temperatures, i.e., 24°C, 5°C, and -20°C. Nearly two log reductions of the *Campylobacter* population were found in skins held at room temperature inoculated with initial bacterial concentrations of 6 log CFU/cm² with an (MOI) of 10. Interestingly, phage levels remained constant over the ten day sampling period, even on skins that went through multiple freeze-thaw cycles, suggesting the temperature stability of certain phages (Atterbury et. al., 2003).

Table 1.1 Summary of phage treatment in the food industry

Food Matrix	Target organism	Log Reductions	Reference
Live chicken	<i>Salmonella</i> Enteritidis	4.2	Atterbury, 2007
Live chicken	<i>Salmonella</i> Typhimurium	2.19	Atterbury, 2007
Chicken litter	<i>Salmonella</i> Typhimurium	1 to 3	Toro, 2005
Calves	pathogenic <i>E. coli</i>	NA	Smith, 1987
Broiler chickens	<i>E. coli</i>	red. mortality	Huff, 2002
Mouse	<i>E. coli</i> O157:H7	1.7 to 2.4	Sheng, 2006
Sheep	<i>E. coli</i> O157:H7	1 to 2	Callaway, 2008
Beef frankfurter	<i>Salmonella</i> DT104	1.8 to 2.1	Whichard, 2003
Chicken skin	<i>Campylobacter jejuni</i>	1 to 2	Atterbury, 2003
Stainless steel	<i>Listeria monocytogenes</i>	3	Hibma, 1997
Honeydew	<i>Listeria monocytogenes</i>	2.0 to 4.6	Leverentz, 2003
Apples	<i>Listeria monocytogenes</i>	> 0.4	Leverentz, 2003
Alfalfa sprouts	<i>Salmonella</i> spp.	1 to 2	Pao, 2004
Cheese	<i>Salmonella</i> Enteritidis	1 to 2	Modi, 2001
Live fish	<i>Streptococcus iniae</i>	red. mortality	Matsuoka, 2007
Beef steak	<i>Pseudomonas</i> spp.	increased shelf life	Greer, 1988

Since phages have a tendency for genetic mutation, there may be a variety of applications for selecting or “breeding” specific phages that are more suitable for certain strains of bacteria. Hibma et. al. (1997) found that bacteriophage specific for the L-form of *L. monocytogenes* could be “bred” to be effective at eliminating biofilm formation on stainless steel while achieving pathogen reduction comparable to lactic acid treatments. This versatility provides a great advantage over conventional chemical treatments in its specificity.

Several studies have focused on the use of bacteriophages directly on fresh-cut fruits and vegetables. Leverentz et. al. (2003) found that bacteriophage mixtures specific

to *L. monocytogenes* reduced bacterial populations on honeydew melon by as much as 4 logs but was much less effective on apples (0.4 log reductions). This suggests that the stability of phage virulence is most likely dependent on certain conditions that may or may not be controllable in some settings. Critics of the study also suggest that it is unlikely for *Listeria* to be present in concentrations as high as 10^5 CFU/ml. In another study sprout seeds were inoculated with a mixture of *Salmonella* Enteritidis, *Salmonella* Montevideo and *Salmonella* Typhimurium (Pao, 2004). Although a mixture of phage was used, reductions of only 1.5 logs were achieved from an initial inoculum of 10^3 to 10^4 CFU/mL. In addition, the study suggested the importance of having multiple phages for each strain of bacteria.

Other studies performed include the application of bacteriophage to inoculated cheese, live fish and beef (Modi et. al., 2001; Matsuoka et. al., 2007; Greer et. al., 1988). Modi et. al. (2001) found 1 to 2 log reductions of *Salmonella* Enteritidis in cheddar cheese with initial bacterial counts of 4 log CFU per gram after addition of phage. A study by Matsuoka et. al. (2007) found significant reductions in mortality of flounder injected with phages specific to *Streptococcus iniae*. Greer et. al. (1988) were able to nearly double the retail shelf life of beef steaks from 3.4 to 6.4 days with phage applied at an (MOI) of 1000.

Challenges to Phage Treatments

One of the major rationales toward the increasing need for alternative forms of antimicrobial treatments is the fact that bacteria have a unique ability to develop resistance to antibiotics as a result of either mutation or the acquisition of foreign DNA. Since bacteriophages eliminate bacteria by attaching to specific cellular receptors on their external membranes, the potential exists for mutation. It has been mentioned in many of the phage studies that the presence of bacteriophage resistant mutants were detected after use of bacteriophage (O'Flynn et. al., 2004; Tanji et. al., 2004). Both O'Flynn et. al. (2004) and Tanji et. al. (2004) conducted experiments to measure the formation of *E. coli* O157:H7 mutants that were resistant to bacteriophage. O'Flynn et. al. determined that while mutations did arise, the frequency was low and the mutants appeared to revert back to susceptible strains after multiple generations. A study by Fischer et. al. (2004) found that because 15% of one particular strain of bacteria were resistant to a specific phage both phage and host could co-exist in continuous culture for days. They indicated the necessity for properly understanding phage-host interactions in a continuous culture before being able to harness phage for other forms of therapy or activity.

Often in order to prevent a bacterial infection from developing resistance to an antibiotic, a combination of antimicrobials will be used in conjunction. Tanji et. al. determined that if a cocktail of bacteriophage being used adhere to unique cell receptor sites, the emergence of resistant mutants was repressed. Another study by Smith et. al. (1987) isolated bacteriophages that adsorbed to the K1 antigen of a pathogenic *E. coli*, assuming that if a mutation occurred that changed the recognition site, the virulence

would be significantly reduced. In experiments involving inoculated mice, they found that the phage cocktail actually worked more effectively than multiple doses of antibiotics. While phage resistant mutants were isolated, they did indeed lack the K1 antigen. Other studies have implicated the necessity for using a phage cocktail to prevent the occurrence of resistant mutants as well (Pao et. al., 2004).

One of the less understood areas surrounding bacteriophage is the factors that are involved in the inactivation of phage, especially under composting conditions. As mentioned earlier, Misra et. al. (2003) determined that properly composted wastes reached sufficient temperatures to inactivate a large range of potential pathogens as well as viruses. A study performed by Johannessen et. al. (2005) tested the survival of a bacteriophage in compost and discovered that its presence could no longer be detected within 3 days even with the addition of the phage's *E. coli* host at several intervals. This however, does not adequately explain the presence of high numbers of bacteriophage persisting in finished compost that has been used as a source of phage for other studies (Greer, 2005).

One of the major challenges present in the compost environment as well as in the food matrix is the low infectious dose of many pathogenic microorganisms. This is particularly devastating for ready-to-eat (RTE) foods and raw produce that is contaminated. Critics of bacteriophage treatment understand that in order for phages to be effective there must be a minimum bacterial density (Wiggins and Alexander, 1984). This can be particularly difficult when treating food products but may be combated in the compost environment. A study done by Lehman, S. (2007) used a non-pathogenic

surrogate of the target organism to sustain phage replication on the surfaces of apples and pears. The phage was then able to propagate in the high numbers of non-infectious bacteria inoculated onto the fruit surfaces, consequently resulting in a large quantity of phage capable of infecting the harmful organisms residing on the surfaces. This concept is especially intriguing because of the abundance of background microorganisms residing in the compost environment, which presents a great potential for finding and developing these phage replication systems.

No adequate studies have been performed to determine the ability of bacteriophage to survive on the surface of open air compost environments. Most organisms however, are to some degree susceptible to the damaging influences of UV radiation. Iriarte et. al. (2006) applied bacteriophage to the surface of tomato leaves at four periods of the day and measured UVA and UVB irradiation. They determined that UV light significantly reduced bacteriophage ability to effectively kill. This presents a challenge to the application of phage on the surfaces of open air compost. Since treatments however would be expected to act quickly on the surfaces, phages infecting bacterial cells would be sheltered until lysis at which point new hosts could be found. Additionally, multiple treatments at key points in the composting process would reduce the concern for phage inactivation followed by pathogen recovery.

Further challenges involve the complex nature of compost materials, the presence of interfering particles as well as inhibitory pH or immobilizing moisture content. Smrekar et. al. (2008) found that bacteriophage T4 lytic activity was not significantly reduced after 4 hours of treatments ranging in pH from 4 to 10, suggesting that the pH is

not a significant factor. Several studies tested the adsorption of enteric phages to clay minerals and found that although phages do adhere to clay particles they do not lose their ability to infect (Schiffenbauer et. al., 1982; Vettori, C. et. al., 1999). Furthermore, the fact that a majority of studies isolate phages by enrichment with sewage, which contains high levels of organics and inhibitors, suggests that a certain level of active phage is maintained at all times regardless of external factors.

Summary

The potential for bacteriophage application is promising for all areas of food safety as well as human therapy. The field itself has not been well explored, only recently gaining recognition for its merits. While there are several critical points that must be addressed when attempting to eliminate the presence of harmful microorganisms from food, the composting environment may be a sufficient, early step in the prevention of issues further down the line. The use of phage in live animals, while worthy of consideration when looking at the health of livestock will never fully eliminate the presence of pathogenic organisms from being shed in feces. There are however a relatively limited number of important organisms in terms of food outbreaks, among which are *Salmonella*, *Campylobacter* and *E. coli* O157:H7. If these organisms were targeted early and re-contamination prevented later, the number of outbreaks would significantly decline. The objectives of this study were as follows:

- Enrichment, isolation and purification of bacteriophages specific to *E. coli* O157:H7 and *Salmonella* spp.

- Characterization of phages by development of host range, visualization and molecular techniques, and optimization of phage cocktails in liquid culture.
- Utilization of phage cocktails to eliminate *E. coli* O157:H7 and *Salmonella* Typhimurium in dairy compost.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE AND DEVELOPMENT OF PHAGE COCKTAILS

Abstract

Thirty-four *Salmonella*-specific and forty-two *Escherichia coli* O157:H7 specific bacteriophages were isolated from raw sewage. Ten *Salmonella*-specific phage strains were selected for further study based on their ability to infect both a wide range of *Salmonella* serotypes and also specific target strains. Initial studies involving phage treatments of *Salmonella* suspended in saline resulted in little or no effect at a multiplicity of infection (MOI) of 1. Several treatment conditions were tested, and it was discovered that phage was most effective against bacteria suspended in tryptic soy broth (TSB), however the presence of phage-resistant mutants led to a resurgence of bacteria. Seven out of 12 challenges resulted in a delay of the onset of exponential growth by as little as 3 h and as much as 10 h. A five-phage cocktail was tested against five strains of *Salmonella enterica* encompassing four serovars: Typhimurium, Poona, Newport and Enteritidis. Of the five phages in the cocktail, electron microscopy revealed that two belonged to the family *Myoviridae* and three to *Siphoviridae*. Restriction digest analysis detected that two of the phages used in the cocktail had identical patterns, however both electron microscopy and host range comparisons yielded similar but non-identical results. The cocktail was effective at preventing the growth of *Salmonella* Typhimurium 8243 indefinitely in TSB. Treatments of the phage cocktail suppressed the growth of other strains for ca. 7 h, however had little effect on preventing their growth. This is most

likely due to the presence of phage-resistant bacteria. The results of this study reveal that it is possible to isolate bacteriophages in abundance from waste water. The optimal conditions for all phages studied were determined to be in TSB, where host bacteria are actively growing. Furthermore, the ability for bacteria to grow in broth can be controlled by the use of specific phage cocktails.

Introduction

Bacteriophages are viruses that infect and destroy bacteria. They are ubiquitous in nature and are estimated to be the most abundant organisms on the planet, numbering in the range of 10^{31} infective virions (Goodridge and Abedon, 2003). Receiving little attention since the early 20th century, phages are now being extensively researched as potential candidates in the fight against bacterial pathogens; especially those with antibiotic resistance. The majority of phage research has focused on the development of molecular techniques, bacterial typing and contamination indication (Hendrix, R.W., 2003; Demczuk et. al., 2003; Mandilara et. al., 2006). They have been successfully implemented to treat human bacterial infections for decades in Eastern Europe. In the U.S. however, recent phage studies have evaluated their effectiveness in live animals (Atterbury et. al., 2007; Smith and Huggins, 1982; Sheng et. al., 2006), fresh produce (Leverentz et. al., 2003; Pao et. al., 2004) and meat products (Whichard et. al, 2003; Greer et. al., 1988). In 2006, the U.S. Food and Drug Administration approved the first phage-based product for control of *Listeria monocytogenes* in food (FDA, 2006). This is just the first among many biotech companies developing phage-based products.

Salmonella spp. and *E. coli* O157:H7 are bacterial pathogens that are commonly associated with outbreaks of food-borne illness (Mead et. al., 1999). Recent outbreaks of *Salmonella* Saintpaul in tomatoes (FDA, 2008) and *E. coli* O157:H7 in spinach (FDA, 2006) have elevated awareness of the need for some ways to eliminate the presence of these organisms in foods. There is a great deal of interest in the potential for bacteriophages to act as a preventive step in the early stages of food production. In order to utilize bacteriophages however, they must be well characterized. The objectives of this study were to isolate and characterize bacteriophages specific to *Salmonella* spp. and *E. coli* O157:H7 using electron microscopy and molecular techniques, and to develop a four or five-phage cocktail effective at eliminating the pathogens *in vitro*.

Materials and Methods

Bacterial strains and culture conditions: Thirteen *Salmonella enterica* subsp. *enterica* strains comprised of serovars Enteritidis ($n=5$), Typhimurium ($n=3$), Newport ($n=2$), Poona ($n=2$) and Heidelberg ($n=1$) were used for bacteriophage enrichment studies. Five rifampin (Fisher Scientific, Fair Lawn, NJ) resistant strains were selected; one from each serovar (two from Typhimurium) for further analysis with bacteriophage isolates. Rifampin resistant bacteria were selected by the use of agar plates containing antibiotics at a concentration gradient. Colonies growing on higher concentrations of antibiotic were chosen and re-streaked until complete resistance was developed. A total of 32 *Salmonella* strains encompassing 20 serotypes were used for host range studies (Table 2.1). Organisms were grown in tryptic soy broth (TSB; Becton Dickinson,

Sparks, MD) with shaking (New Brunswick C25 incubator shaker; Edison, NJ) overnight at 37°C to early log phase. The cells were washed in 0.85% saline to an optical density, at 600 nm wavelength, of 0.5 as determined by a spectrophotometer (µQuant; Bio Tek, Winooski, VT). Further dilution with saline was used to produce the necessary concentrations.

Enrichment and isolation of bacteriophage: Bacteriophages were isolated specific to *Salmonella* strains using a total of five samples of wastewater collected from treatment facilities in Pendleton, Anderson and Clemson, SC. Activated sludge and, when available, raw influent were collected in 250 ml bottles and stored on ice for 2-4 h before processing. To 5 ml of double strength TSB (DS TSB) was added 100 µl of each host strain and the volume was brought to 10 ml with a sewage mixture. The enrichment tubes were incubated overnight at 37°C, and the following day, the liquid was centrifuged at 2,500 x g for 10 min and the supernatant was filtered through a 0.22 µm filter (Nalgene, Rochester, NY).

A 10 µl drop of phage suspension was pipetted onto a tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) plate overlayed with 3 ml of 0.6% agar (Becton Dickinson, Sparks, MD) containing a single *Salmonella* strain at a concentration of 10⁶ CFU/plate. Bacterial strains that had a clearing zone were then tested against several dilutions of phage stock by performing a soft agar overlay method. A 100 µl suspension of bacteriophage was mixed with 100 µl of log phase bacteria at 10⁷ CFU/ml for 10 min in a 96-well microplate (96 Well Cell Culture Cluster, Corning, NY). The mixture was then transferred into 3 ml of 0.6% agar and overlayed onto TSA. Plaques were isolated

by using a 1 ml pipette tip, sterilely cut with scissors, and were suspended in 1 ml of SM buffer [100 mM NaCl, 8 mM MgSO₄*7H₂O, 50 mM Tris-Cl (pH 7.5)]. Following overnight incubation at 4°C plaque purification was repeated 2 times. Phage stock solutions were prepared by performing soft agar overlays of multiple phage dilutions. The plate that cleared all bacterial growth at the lowest concentration of phage was washed with 10 ml of SM buffer at 4°C overnight. The surface liquid was removed and filtered.

Bacteriophage FO was kindly provided by the University of Calgary and a stock solution was prepared as described above. Phages specific to *E. coli* O157:H7 (E0654, E01993, E009 and SPp) were isolated previously from another project, and were characterized and used solely in compost trials.

High titer bacteriophage preparation: In preparation for high concentrations of phage, the host strain was grown to log phase in 10 ml of TSB. The turbid culture was used to inoculate two flasks of 500 ml TSB and incubated at 37°C for 1 h. Phage was added at an MOI of 0.1 and the mixture incubated with shaking until turbidity was cleared (approximately 3 h). The broth was centrifuged in Beckman J2-HS (Beckman, Schaumburg, IL) at 5,000 x g for 10 min and the supernatant was poured into a centrifuge tube containing 20 g of NaCl and 50 g of Polyethylene glycol 6000 (PEG 6000; USB Corporation, Cleveland, OH). The mixture was inverted until dissolved and stored at 4°C overnight. On the following day, the tubes were centrifuged at 15,000 x g for 30 min and the supernatant was removed. The precipitate was re-suspended in 200 ml of SM buffer and the duplicate samples were pooled. Sixteen grams of NaCl and 40 g of PEG 6000

were added and dissolved overnight at 4°C. Tubes were centrifuged at 15,000 x g for 30 min and the supernatant was removed. The precipitate was dissolved in 5 ml of SM buffer and 5 ml of chloroform and the mixture stored at 4°C overnight. Centrifugation was then performed at 5,000 x g for 10 min and the aqueous phase collected and filtered as a high titer stock (generally $10^{10} - 10^{12}$ PFU/ml).

Host range determination: Host range was determined for 34 strains of phage against 32 strains of *Salmonella* comprised of 20 unique serotypes. Host strains were overlayed in 3 ml of 0.6% agar onto TSA at a concentration of 10^6 CFU/plate. Phage was added at an MOI of 1 in 10 µl drops. Plates were incubated overnight at 30°C. The minimal phage concentration required for complete lysis was determined by pipetting 10 µl drops of phage stock diluted from 10^{-2} to 10^{-10} onto the surface of TSA overlayed with 0.6% agar containing one *Salmonella* strain. It was determined that based on the area of a 10 µl drop of phage suspension ($2.9 \text{ cm}^2 \pm 0.74 \text{ cm}^2$), and a bacterial inoculum of 10^6 CFU/plate, each spot would cover an average of 10^4 CFU's. The estimated number of bacterial cells within the area of a 10 µl drop was then compared with the lowest dilution of phage that would lyse all bacteria, resulting in the minimal MOI necessary for clearing.

Transmission electron microscopy: Copper grids (400 mesh, EMS, Hatfield, PA) were coated with 0.5% formvar dissolved in dichloroethane (Fischer, Fairlong, NJ). Dried grids were placed on parafilm (American National Can, Menasha, WI) and a 5 µl drop of phage solution (10^{10} PFU/ml) pipetted on the surface for 1 min. The liquid was drawn off with filter paper and the grid stained for 30 seconds with a 5 µl drop of 0.5%

phosphotungstic acid (PTA; EMS, Hatfield, PA) or 2% uranyl acetate (UA; EMS, PA). Grids were air dried and viewed on a Hitachi H-7600 electron microscope (Hitachi, Tokyo, Japan) at 120-keV accelerating voltage.

DNA isolation and restriction endonuclease analysis: Fresh phage stocks were prepared as described above prior to DNA extraction. Qiagen Lambda mini kit (Qiagen, Valencia, CA) and the MoBio Ultra Clean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) were compared for procedure time versus quality of DNA. To determine whether the MoBio kit was successful at isolating pure phage DNA, an additional 30 minute incubation at 37°C with DNaseI and RNaseA (Qiagen Buffer L1) treatment was compared to the normal MoBio procedure. DNA samples were analyzed in 1.5% agarose (Bio-Rad, Hercules, CA) run at 50 V and stained with ethidium bromide for 20 min followed by a 10 min de-staining in distilled water.

DNA samples from 5 selected *Salmonella* phages and 4 *E. coli* O157:H7 phages were digested with the restriction endonucleases DraI and NdeI (Promega, Madison, WI) according to the supplier's recommendations. DNA fragments were separated by electrophoresis in 1.5% agarose gels in 1X Tris-borate-EDTA buffer, at 50 V with a Wide Mini-Cell® Sub GT agarose gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA).

***In vitro* phage optimization studies:** Several approaches were tested to optimize the phage lytic activity against pathogens.

Initial saline trial: The ten phages selected from host-range experiments were initially tested against five *Salmonella* strains representing four serotypes (Table 2.1).

Phage stock solutions were warmed at 37°C for 1 h to reduce clumping. Bacterial strains were prepared as mentioned above and diluted to 10^4 CFU/ml in a 96-well plate. Phages were diluted and added at an MOI of 1 with control wells containing bacteria and SM buffer. After 3 and 8 h, 50 µl samples were spiral plated (Autoplate®4000, Bethesda, MD) onto TSA and incubated at 30°C overnight. The following day colonies were counted.

Well blocking trial: Phage H9301 (1)-2 was tested in duplicate in a 96-well plate against *Salmonella* Enteritidis H2292. One plate was blocked by filling wells with 350 µl of 1% peptone (Becton Dickinson, Sparks, MD) and incubated for 2 h at 37°C followed by three washes with sterile distilled water. Initial bacterial concentrations of 10^3 , 10^4 , and 10^5 CFU/ml were inoculated into either 0.85% saline or TSB. Bacteriophage was added at an MOI ranging from 0.001 to 100 (Table 2.2). Fifty microliter samples were taken at 2 and 4 h and spiral plated onto TSA. Optical density of wells containing TSB was measured at 0, 2, 7 and 16 h using a spectrophotometer.

Optimal media trial: To determine the most effective environment for *in vitro* phage application, *Salmonella* Enteritidis H2292 was diluted and added into blocked or unblocked 96-well plates containing either 0.85% saline, SM buffer or TSB at a concentration of 10^5 CFU/ml. Bacteriophage H9301 (1)-2 was added at an MOI of 0, 0.1, 1 or 10. Fifty microliter cell suspensions were spiral-plated after 15 min, 2 and 4 h. Optical density measurements were taken for the wells containing TSB at intervals between 0 and 24 h.

Development of phage cocktail: Five phages selected for use in a cocktail were tested individually in TSB supplemented with rifampin (TSBR) against their respective *Salmonella* host (those that were cleared by minimal inhibitory MOI assay). Bacterial cultures were diluted to 10^4 CFU/ml and challenged with bacteriophage at MOIs listed in Table 2.3. The optical densities were measured at 0, 9, 13, 15 and 22 h. A cocktail of five phages was used at the same ratios in Table 2.3 against each of the five *Salmonella* strains diluted to 10^6 , 10^7 , and 10^8 CFU/ml in TSB supplemented with rifampin (TSBR). Optical densities were measured every hour for nine hours followed by a second phage treatment performed by transferring 200 μ l of duplicate samples into 100 μ l of fresh phage cocktail. Controls were transferred into 100 μ l of fresh SM buffer. Measurements were taken up to 24 h and visual observations made as far as 72 h.

Results and Discussion

Bacteriophage isolation and host range determination

Bacteriophages were isolated from raw sewage for ten of the thirteen *Salmonella* strains used as host for enrichment (Table 2.1). Phages were most commonly isolated for strains of *Salmonella* Enteritidis. This was not surprising, considering that *Salmonella* Enteritidis was ranked among the top two strains most commonly associated with salmonellosis in humans from 1987 to 1997 (Olsen et. al., 2001). Additionally, five *Salmonella* Enteritidis strains were used in phage enrichment studies and may have out-competed some of the other serotypes resulting in a larger population of *Salmonella* Enteritidis-specific phages.

Host range comparison revealed that the majority of phages were able to lyse *Salmonella* strains used during the initial enrichment, with six being able to infect more than 10 of the 32 strains, but none effective against all strains (Table 2.4). Bacteriophage FO had a unique host range, being able to lyse ten serotypes. One important characteristic of phage FO is that it has been previously characterized (Kuhn, J., 2002) and is propagated in *Salmonella* Typhimurium. This came in useful since few effective phages were isolated, in this study, that were able to infect the virulent form of *Salmonella* Typhimurium (DT104). Visual comparison of the host ranges led to the preliminary selection of ten strains of bacteriophage (Table 2.4). Some phages were chosen based on their ability to lyse multiple serotypes, while others were chosen because they infected specific strains of *Salmonella*. For instance, phages H2292{2} and H4717 S5p2, effective against *Salmonella* Enteritidis, *Salmonella* Newport and *Salmonella* Typhimurium, also lysed five and four other serotypes, respectively. Bacteriophage ME-18 p2 on the other hand was more effective at killing only two serotypes, *Salmonella* Enteritidis and *Salmonella* Typhimurium. Few phages were able to lyse *Salmonella* Typhimurium DT104, therefore several were chosen specific to that strain regardless of their lytic activity toward other *Salmonella* serotypes.

To access a broader range of phages it may be necessary to get multiple samples from a variety of human and animal wastes. McLaughlin et. al. (2006) discovered the presence of large numbers of phage lytic to *Salmonella* Typhimurium strains when enrichments were performed from swine manure. A study by Carey-Smith et. al. (2006) on *Salmonella* phages isolated multiple phages from sewage specific to *Salmonella*

Typhimurium as well as *Salmonella* Enteritidis suggesting that either not enough *Salmonella* Typhimurium strains were used for enrichment, or phage populations were variable in our study.

MOI testing was performed on TSA plates to narrow the potential phages down to five strains to be used in a cocktail. The MOIs for each bacteriophage against the five *Salmonella* strains were scored based on their effectiveness (Table 2.3). Scores were determined as follows: phages with an optimal MOI of 0.001 were given a six, while those with an MOI of 100 were given a 1. Numbers were assigned sequentially for MOIs that fell in between and the totals summed. It was assumed based on preliminary restriction analysis that p2 and p4 were identical phages, therefore p2 was arbitrarily chosen for further study. The final phages selected for *Salmonella* treatment in the following experiments were H2292{2}, H9301 (1)-2, FO, ME-18 p2 and H4717 S5p2.

Electron microscopy and restriction analysis

Transmission electron microscopy images of the five *Salmonella* phages (Fig. 2.1-2.5) revealed that all had unique morphologies. The presence of a contractile tail for both H4717 S5p2 and FO suggest that they belong to the family of *Myoviridae*. Phage H4717 S5p2 had an elongated head of 93 nm with a tail length of 95 nm, while FO had more standard dimensions of head length, 62 nm, and tail length, 116 nm. Phages ME-18 p2, H9301 (1)-2 and H2292{2} had flexible non-contractile tails and were of the family *Siphoviridae*. Phage H2292{2} had a tail that was nearly 229 nm, while ME-18 p2 and H9301 (1)-2 had tail lengths of 102 and 151 nm, respectively. Four phages specific to *E. coli* O157:H7 strain 0923-21 were also analyzed by restriction enzyme digestion and

electron microscopy. Phage strain E0654 had an isometric head with a diameter of 78 nm, tail diameter of 15-nm and length of 125-nm (Fig 2.6). Phages E0654, E009 and E01993 (not shown) were classified as *Myoviridae*.

Restriction analysis by NdeI of the five *Salmonella* phages revealed that phages H9301 (1)-2 and H2292{2} share the same patterns, whereas the rest of the phages appeared to have unique patterns. Further digestion with DraI confirmed that H9301 (1)-2 and H2292{2} have identical patterns (Fig. 2.7), however host range analysis revealed differences among the two. Additionally, phage H2292 {2} had an unusually long tail (229 nm) when compared with H9301 (1)-2 (151 nm). These two phage strains, isolated from different sources could be closely related but diverged enough to change host range. Heineman et. al. (2008) found that both host range and adsorption rate are genetically determined and that changes in host range are common. Tetart et. al. (1996) observed that small duplications in the tail fiber of phage T4 allowed it to incorporate *Yersinia pseudotuberculosis* into its host range.

A total of forty-two *E. coli* O157:H7 specific phages were isolated in the same way as *Salmonella* using 14 strains of *E. coli* O157:H7 as host. To ensure that the phages in the phage cocktail specific to *E. coli* O157:H7 were all unique, restriction analysis was performed and revealed that strains E0654 and E009 had similar patterns, although neither was cut well with DraI or NdeI (data not shown). Phages E0654 and E009 were also classified as *Myoviridae*. Phages E01993 and SPp however, yielded unique band patterns upon digestion with each of the enzymes.

In vitro phage studies and cocktail development

Bacteriophage host range analysis on TSA provided enough information to select a working group of ten bacteriophages for assays in liquid. An initial host range study with each bacteriophage at an MOI of 1 against five individual *Salmonella* strains resulted in little to no reductions in bacterial numbers (data not shown). Similar trials on TSA at the same MOI resulted in complete lysis and plaque formation for a majority of the phage-bacteria combinations (Table 2.4). Only bacteriophages H3353 S6p2 and H3353 p1 had significant reductions of ca. 1 log against *Salmonella* Typhimurium DT104, and 1 and 3 logs against *Salmonella* Enteritidis H2292 and *Salmonella* Typhimurium 8243, respectively. Restriction analysis revealed that H3353 p1 and S6p2 were identical strains.

The lack of effect of the majority of bacteriophages on all *Salmonella* strain tested is not entirely surprising due to the physiological state of the host organisms in a saline solution. Heineman et. al. (2008) reported on the importance of the bacterial physiological state in determining bacteriophage adsorption. There is also the potential for phage interaction with bacterial cells without the occurrence of adsorption, perhaps due to a lack in particular cofactors. Suarez et. al. (2008) found that certain strains of *Lactococcus* phage required calcium ions to complete a lytic cycle, however it was not shown to be necessary for efficient phage adsorption. The potential also exists for bacteriophage replication to be aborted as a result of the presence of extra-chromosomal elements (Duckworth et. al., 1981), which are typically found in *Salmonella* strains (Cormican et. al., 2002).

One possible explanation for the success of phages H3353 S6p2 and p1 could be a higher initial concentration of phage, resulting in lysis of bacterial cells by cellular disruption due to massive infection (Tarahovsky et. al., 1994). The host range test for this particular phage showed that it had an incomplete effect on *Salmonella* Enteritidis H2292 and even less of an effect on *Salmonella* Typhimurium DT104 on agar, which may suggest that it is more effective in liquid. Another possibility, although unlikely, is the presence of residual chloroform from the stock preparation that could have resulted in *Salmonella* death.

A study by McLaughlin et. al. (2008) found that bacteriophage titers declined in 96-well microplates due to phage adsorption to the well surfaces, while not affecting *Salmonella* numbers. They were able to eliminate this by blocking the wells with a pre-treatment of 1% peptone. To determine whether a similar problem was occurring in this study, one experiment using phage H9301 (1)-2, known to form plaques against *Salmonella* Enteritidis H2292, was performed in 0.85% saline at MOIs ranging from 0.001 to 100 (Table 2.2). There was no difference in plate counts between controls and any of the phage MOIs at three different initial concentrations of bacteria suggesting that either the blocking was insufficient or other factors were inhibiting phage in saline.

To test whether the medium was playing a role in phage effectiveness, an experiment was performed using saline, SM buffer and TSB with both blocked and unblocked 96-well plates. Phage H9301 (1)-2 was again tested against *Salmonella* Enteritidis H2292, at MOIs of 0 (control), 0.1, 1, and 10. Figures 2.8a-c reveal that no lytic effect was observed in either saline or SM buffer after 2 and 4 h, however an almost

one log reduction occurred in the treatments in TSB containing a phage to bacteria ratio of 10 and 1. Some reduction was seen at an MOI of 0.1 as well. Although the levels of reduction were not high, it could be confirmed that bacterial lysis was occurring in the broth as opposed to two buffer solutions. The most likely reason for this is that the bacterial physiology in TSB is more suitable for phage infection. Furthermore the presence of an undefined media increases the likelihood that any molecules that may play a key role in phage adsorption or replication would be available. Therefore all further experiments were performed in TSB supplemented with rifampin (TSBR) to prevent any risk of contamination.

A phage study was performed in TSB on each of the five *Salmonella* strains using the optimal MOI data from Table 2.3. For example, bacteriophages FO and S5p2 were tested separately in TSBR against *Salmonella* Typhimurium DT104 at MOIs of 0.01 and 100, respectively (Table 2.3). S5p2 treatment was no different from the control growth curve, while FO delayed bacterial growth by several hours (data not shown). None of the individual bacteriophages were capable of preventing bacterial growth permanently, however 7 out of 12 challenges resulted in a delay of the onset of exponential growth by as little as 3 h and as much as 10 h. Phage H4717 S5p2 prevented the growth of *Salmonella* Enteritidis H2292 for 10 h. ME-18 p2 also prevented *Salmonella* Enteritidis from growing for 8 h but had no effect on *Salmonella* Typhimurium 8243. H9301 (1)-2 however, prevented *Salmonella* Typhimurium 8243 from growing for 7 h and also had an effect on Newport H9116. Only bacteriophage FO suppressed the growth of *Salmonella* Typhimurium DT104 for a significant period of time. The discrepancies between liquid

assays and plate assays suggest the importance of understanding how certain bacteriophages act under different conditions.

Similar studies by McLaughlin, M.R. (2007) and O'Flynn et. al. (2004) found that in most situations, single phages are insufficient to eliminate an entire bacterial population in broth, highlighting the importance of developing phage cocktails that can prevent the development of bacteriophage-insensitive mutants (BIM's). O'Flynn et. al. (2004) developed a three phage cocktail for the elimination of *E. coli* O157:H7 and found that initial inoculums of 10^5 CFU/ml were reduced to zero within one hour following a phage challenge with an MOI between 1 and 100. However within one more hour bacterial numbers re-grew to nearly 3 logs.

Phages H2292{2}, H9301 (1)-2, H4717 S5p2, ME-18 p2 and FO were made into a cocktail at MOIs determined by plate assay for *Salmonella* Typhimurium 8243, which was to be used for compost studies. All five *Salmonella* strains were tested in TSBR against the phage cocktail (Figures 2.9a-e). The results were nearly identical for *Salmonella* Enteritidis H2292, *Salmonella* Newport H9116 and *Salmonella* Poona H9301, in which bacterial growth was suppressed for about 7 h relative to controls. Although bacterial growth did occur, it never reached the absorbance of the controls within the 24 h sampling period. The two strains of *Salmonella* Typhimurium had completely opposite results with DT104 growth being suppressed for several hours followed by re-growth above that of the controls. *Salmonella* Typhimurium 8243 had a brief weak peak of growth at 17 h but quickly fell back to zero. Visual observations at 72 h confirmed that the broth remained clear, suggesting that the cocktail completely

eliminated *Salmonella* Typhimurium 8243. Subsequent phage treatments after 10 h did not reduce numbers or growth rates in any of the experiments suggesting that fresh bacteriophage is still not capable of infecting the mutant bacterial strains. Reductions in optical density during the second treatment were only a result of dilution of the samples into SM buffer or phage.

The fact that only one strain of *Salmonella* was completely inhibited by the phage cocktail emphasizes the importance of developing cocktails that contain several phages specific to each of the strains being tested. Analysis of Table 2.3 reveals that four of the five phages used in the cocktail successfully cleared plates of *Salmonella* Typhimurium 8243, which is more than any of the other strains and consequently the reason that the cocktail was more effective for that strain.

Conclusions

This study provides conclusive evidence of the fact that bacteriophages specific to *Salmonella* and *E. coli* O157:H7 can be found in abundance in human waste. Both TEM and restriction digest assays can be used to characterize and identify certain phages that can be developed into a cocktail that is effective at eliminating specific bacterial strains. Further research with optimal multiplicities of infections as well as infection conditions would result in more effective phage assays. The relative abundance of phages in nature allows for the potential of developing a bacteriophage library that could be used to create cocktails custom-tailored to specific bacteria of interest. However, the effectiveness of a phage cocktail in broth or on plate is of little use if it is not optimized for treatments in

real samples. Trials will need to be performed utilizing the phage cocktail tested here in many different areas of the food production chain.

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Figure Legend

Figure 2.1-2.6: Transmission electron micrographs of *Salmonella*-specific phages: H4717 S5p2 (2.1) observed at 346,000X; ME-18 p2 (2.2) at 333,000X; FO (2.3) at 354,000X; H9301 (1)-2 (2.4) at 324,000X; H2292{2} (2.5) at 459,000X and *E. coli* O157:H7-specific phage E0654 (2.6) at 351,000X. Phages were stained with either phosphotungstic acid or uranyl acetate.

Figure 2.7: Restriction analysis of five *Salmonella* phage isolates using enzyme DraI. **Lane M**, Lambda HindIII/EcoRI DNA marker; **Lane 1-2**, FO DNA and digest; **Lane 3-4**, H9301 (1)-2 DNA and digest; **Lane 5-6**, H4717 S5p2 DNA and digest; **Lane 7-8**, H2292{2} DNA and digest; **Lane 9-10**, ME-18 p2 DNA and digest.

Figure 2.8a-c: Optimal media for phage infection was tested in saline (a), SM buffer (b) and TSB (c), with duplicate wells blocked with 1% peptone (— — —) and those without (— — —). Bacteriophage H9301 (1)-2 known to be effective against *Salmonella* Enteritidis H2292 was inoculated at MOIs of 0 (◇), 0.1 (○), 1 (Δ), and 10 (□) in TSB.

Figure 2.9a-e: The effect of phage cocktail applied to *Salmonella* Typhimurium 8243 (a), *Salmonella* Enteritidis H2292 (b), *Salmonella* Newport H9116 (c), *Salmonella*

Typhimurium DT104 (d) and *Salmonella* Poona H9301 (e) at an initial concentration of 10^8 CFU/ml in TSB. Controls (—) and treatments (—) for the single cocktail phage trial were measured every hour for nine hours. At 10 h, duplicate samples of controls (—) and treatments (—) were added to a second round of SM buffer or phage, respectively. The time point where a second treatment was performed is marked with an arrow.

Table 2.1 *Salmonella enterica* strains used for phage enrichment and host range study

Serovar	ID	origin	no. phages isolated
Enteritidis	Benson ^a		4
	H2292 ^b	Dr. Doyle (UGA)	5
	H3353		5
	H4717		3
	ME-18		5
Typhimurium	ATCC 14028		2
	Copenhagen		— ^c
	DT104 ^b	ATCC 700408	1
	8243 ^b	Dr. Curtis (WSU)	1
Newport	H9113		0
	H9116 ^b		2
Poona	H9301 ^b		5
Heidelberg	UNL		0
Amsterdam	155A	isolate ^d	—
Banana	222	isolate	—
Demerara	223B	isolate	—
	193B	isolate	—
Idikan	98	isolate	—
IIIa	145	isolate	—
Johannesburg	161A	isolate	—
Livingstone	6B5	isolate	—
Mbandaka	214	isolate	—
	226	isolate	—
Molade	65A1	isolate	—
Montevideo	62A1	isolate	—
	65B2	isolate	—
Orangienburg	6A3	isolate	—
Putten	189A	isolate	—
Rough	156A	isolate	—
Seftenburg	122	isolate	—
	18A3	isolate	—
Tennessee	K4920	isolate	—

^a Bolded strains used for phage enrichment^b Strains selected for *in vitro* phage studies^c Strains used solely for phage host range study^d Isolates from rendered animal meats and compost

Table 2.2 Experimental design for MOI ranges

Phage (PFU/ml)	Bacterial strain (CFU/ml)		
	10^5	10^4	10^3
10^5	1	10	100
10^4	0.1	1	10
10^3	0.01	0.1	1
10^2	0.001	0.01	0.1

Table 2.3 The minimal inhibitory MOI on agar for phage isolates against *Salmonella* strains

Serotype and strain	MOI for complete lysis for phages									
	{2}	(1)-2	FO	p2	p4	S6p2	S5p2	p1	S5p1	S6p1
Enteritidis H2292				0.01 ^a	0.01	0.01	0.01	1	0.001	
Newport H9116	1	0.1	10							
Poona H9301	0.1	0.1								
Typhimurium 8243	0.001	0.01	0.01	0.001	0.001	0.001	1	0.001	100	1
Typhimurium DT104			0.01				100			
Score:	13^b	13	12	11	11	11	9	9	7	3

^aMOIs were scored as follows: 0.001, 6; 0.01, 5; 0.1, 4; 1, 3; 10, 2; 100, 1

^bBolded numbers represent phages selected for cocktail

Table 2.4 Host range of 10 *Salmonella* bacteriophage isolates determined on 34 *Salmonella* host strains^a

<i>S. enterica</i> serotype or strain	Lysis by bacteriophage:									
	S5p1	S5p2	S6p1	S6p2	p1	p2	p4	FO	(1)-2	{2}
Amsterdam	S	++	S	S	-	-	-	++	-	++
Banana	-	-	-	-	-	-	-	-	-	-
Demerara (193B)	-	-	-	-	-	-	-	-	-	-
Demerara (223B)	-	-	-	-	-	-	-	+	-	-
Enteritidis (Benson)	+++	+++	+	+++	+++	+++	+++	S	++	++
Enteritidis (H2292)	+++	+++	-	++	++	+++	+++	-	++	++
Enteritidis (H3353)	+++	+++	+	+++	+++	+++	+++	++	+++	+++
Enteritidis (H4717)	+++	+++	+	+++	+++	+++	+++	+++	+++	+++
Enteritidis (ME18)	+++	+++	+	+	+	+++	+++	+++	+++	+++
Heidelberg	-	S	-	-	-	-	-	++	++	++
Idikan	-	-	-	-	-	-	-	++	S	-
IIIa	-	-	-	-	-	-	-	-	-	-
Johannesburg	S	-	S	++	-	-	-	-	+++	+++
Livingstone	-	-	-	-	-	-	-	-	-	-
Mbandaka (214)	-	-	-	-	-	-	-	-	-	-
Mbandaka (226)	-	-	-	-	-	-	-	+	+++	+++
Molade	-	-	-	-	-	-	-	-	-	-
Montevideo (62A1)	++	++	-	++	-	-	-	S	-	-
Montevideo (65B2)	S	++	-	++	-	-	-	S	-	-
Newport (H9113)	++	++	-	++	-	-	-	S	++	++
Newport (H9116)	+++	+++	+++	+++	+	-	+++	+++	+++	+++
Orangienburg	S	++	S	++	-	-	-	++	-	-
Poona	S	S	-	S	-	-	-	-	-	-
Putten	-	-	-	-	-	-	-	+	S	S
Rough	-	-	-	-	-	-	-	-	-	-
Seftenburg (18A3)	S	++	-	S	-	-	-	-	-	S
Seftenburg (122)	++	++	-	++	-	-	-	-	-	++
Tennessee	-	-	-	-	-	-	-	-	-	-
Typhimurium (8243)	++	+++	+++	+++	+++	+++	-	S	++	+++
Typhimurium (ATCC 14028)	S	++	+	+++	+++	++	S	S	-	S
Typhimurium (Copenhagen)	++	++	-	S	++	++	++	++	-	S
Typhimurium (DT104)	++	++	-	S	++	++	++	++	-	S
Total no. of strains lysed	12	17	7	14	9	9	8	12	11	13

^aA subset of the host range data obtained for 34 bacteriophage isolates screened against 34 *Salmonella* strains. Results were recorded as follows: +++, confluent lysis; ++, semiconfluent lysis; +, individual plaques; S, shadow lysis; -, no lysis.

Table 2.5 Comparative analysis of lytic effects of bacteriophage on agar^a and in TSB

Serotype and strain	Phage strains				
	{2}	(1)-2	FO	p2	Ssp2
Enteritidis H2292				+/+ ^b	+/+
Newport H9116	+/-	+/+	+/-		
Poona H9301		+/+			
Typhimurium 8243	+/+	+/+		+/-	
Typhimurium DT104			+/+		+/-

^aAgar results are from Table 2.3

^b+/+, positive for lysis on agar and in TSB; +/-, positive for lysis on agar but not in TSB

Figure 2.1

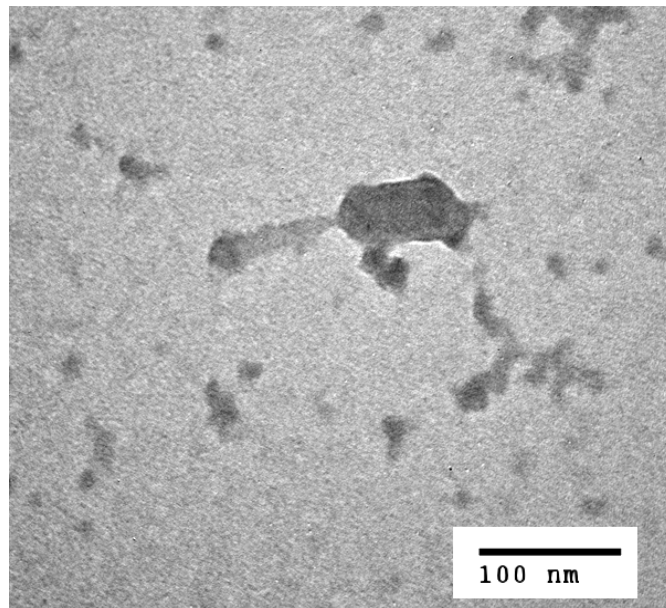


Figure 2.2

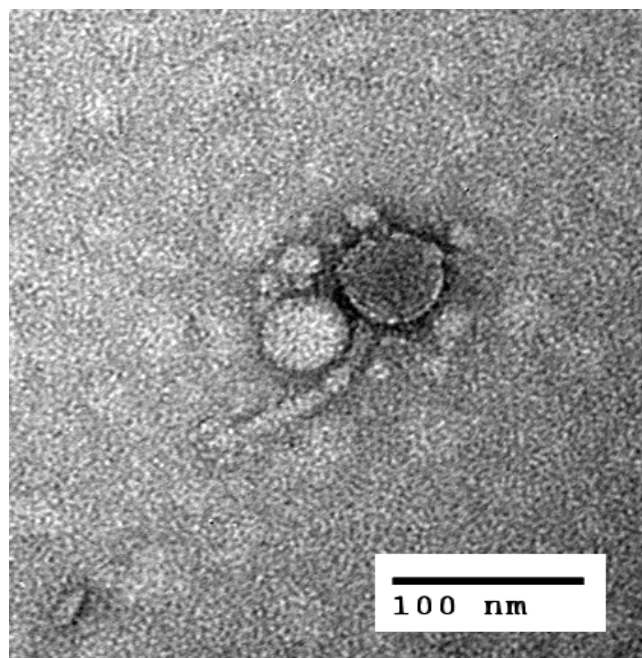


Figure 2.3

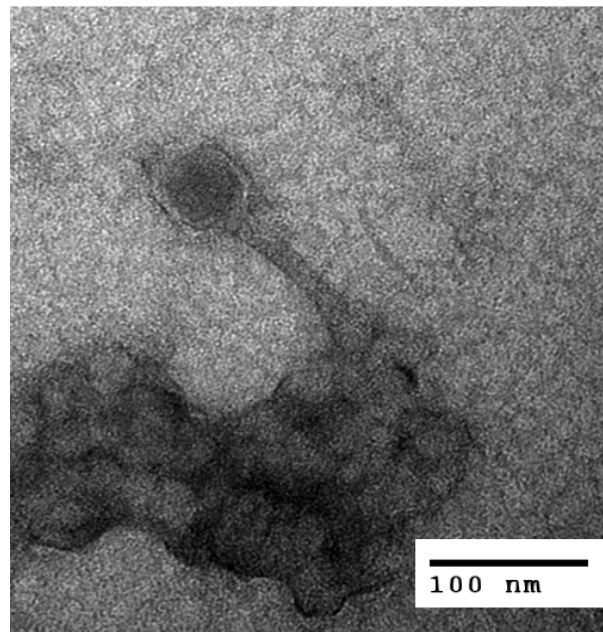


Figure 2.4

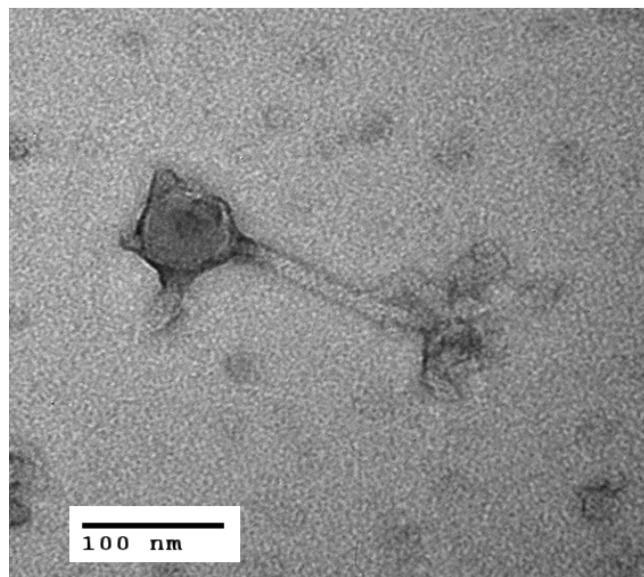


Figure 2.5

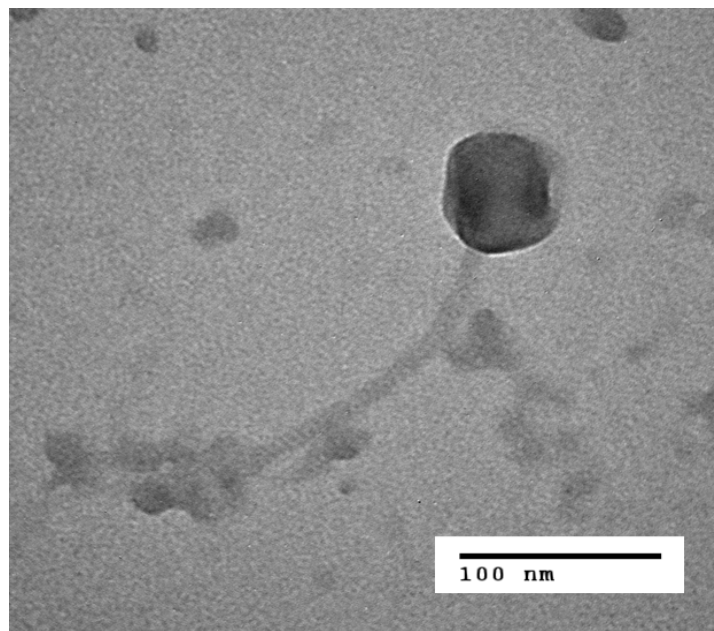


Figure 2.6

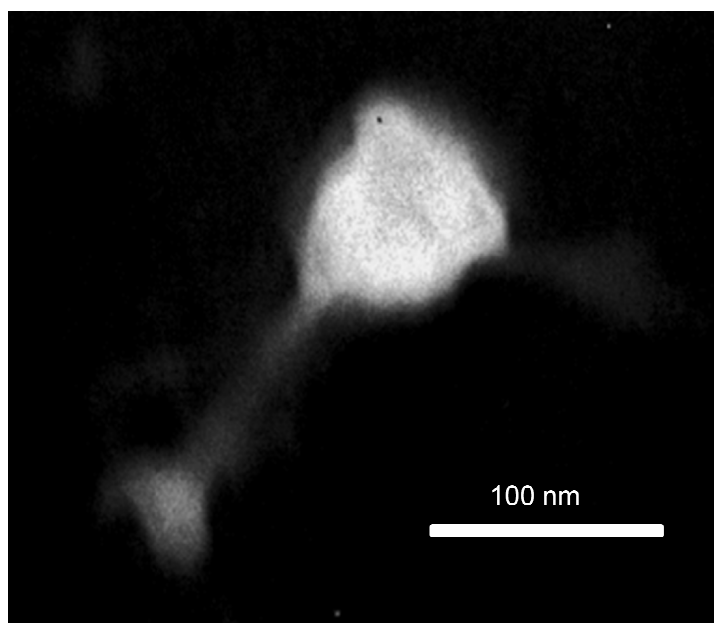


Figure 2.7

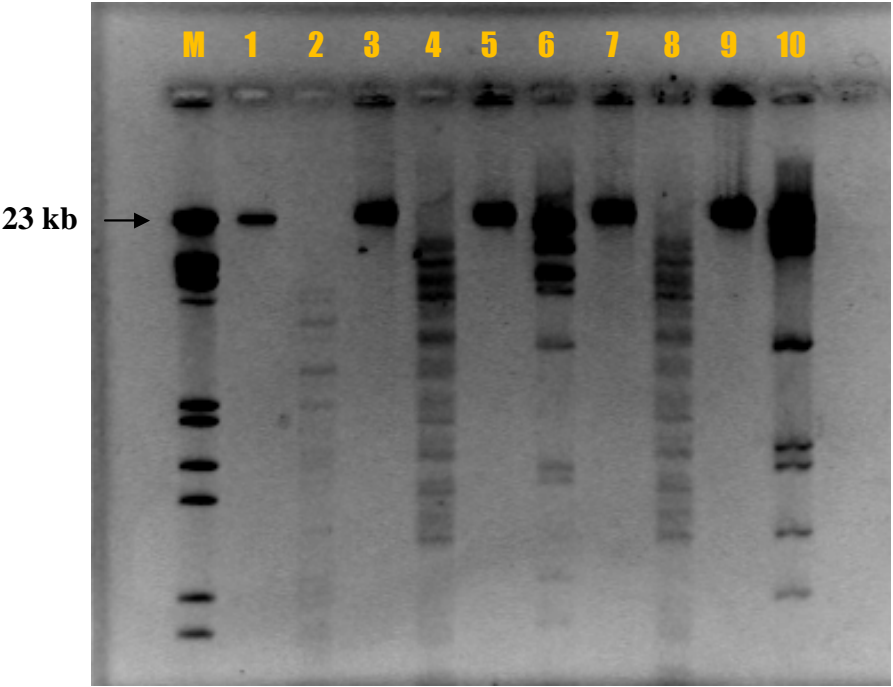


Figure 2.8a

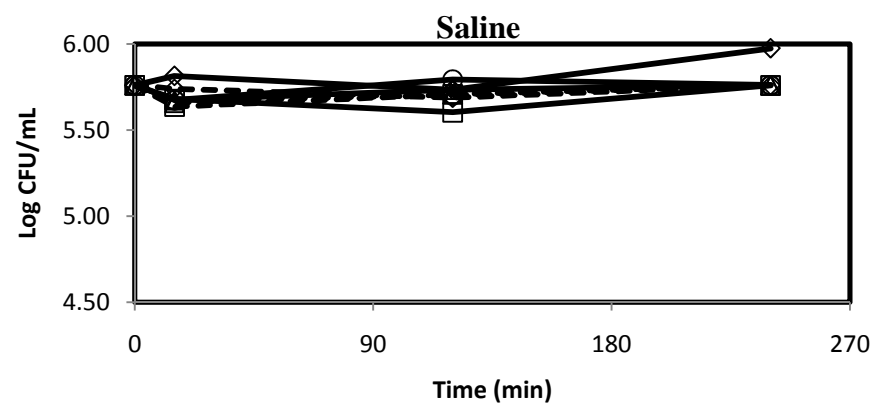


Figure 2.8b

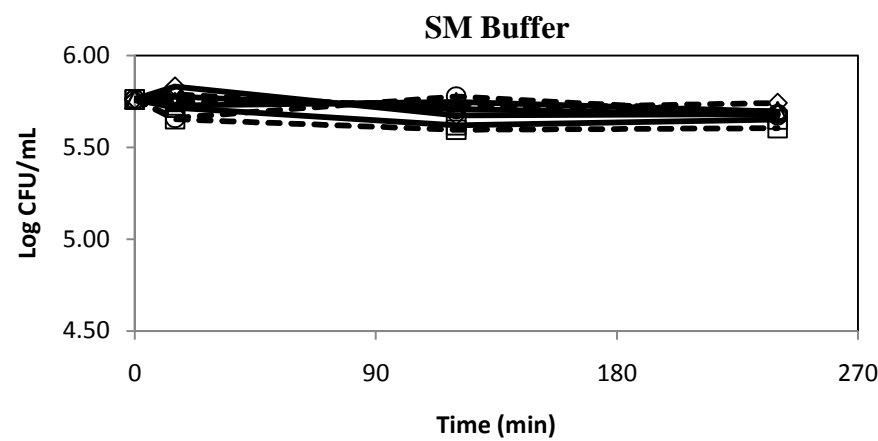


Figure 2.8c

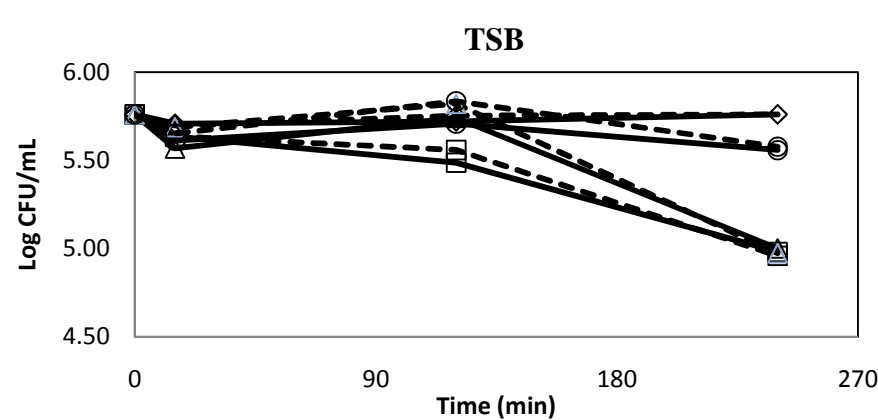


Figure 2.9a

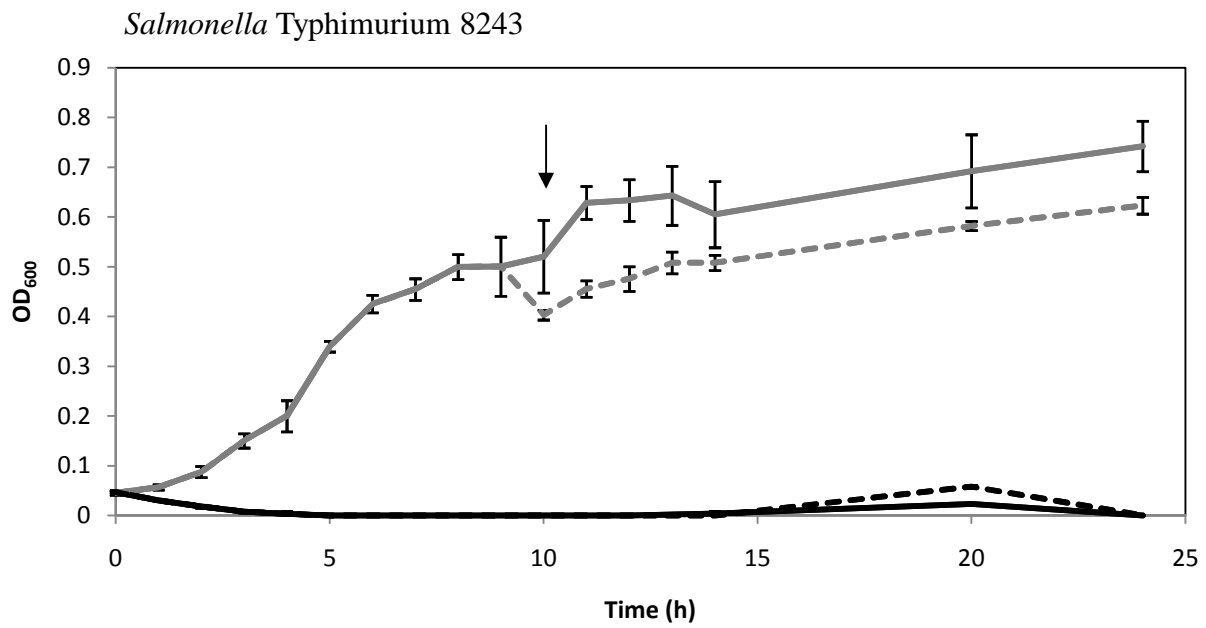


Figure 2.9b

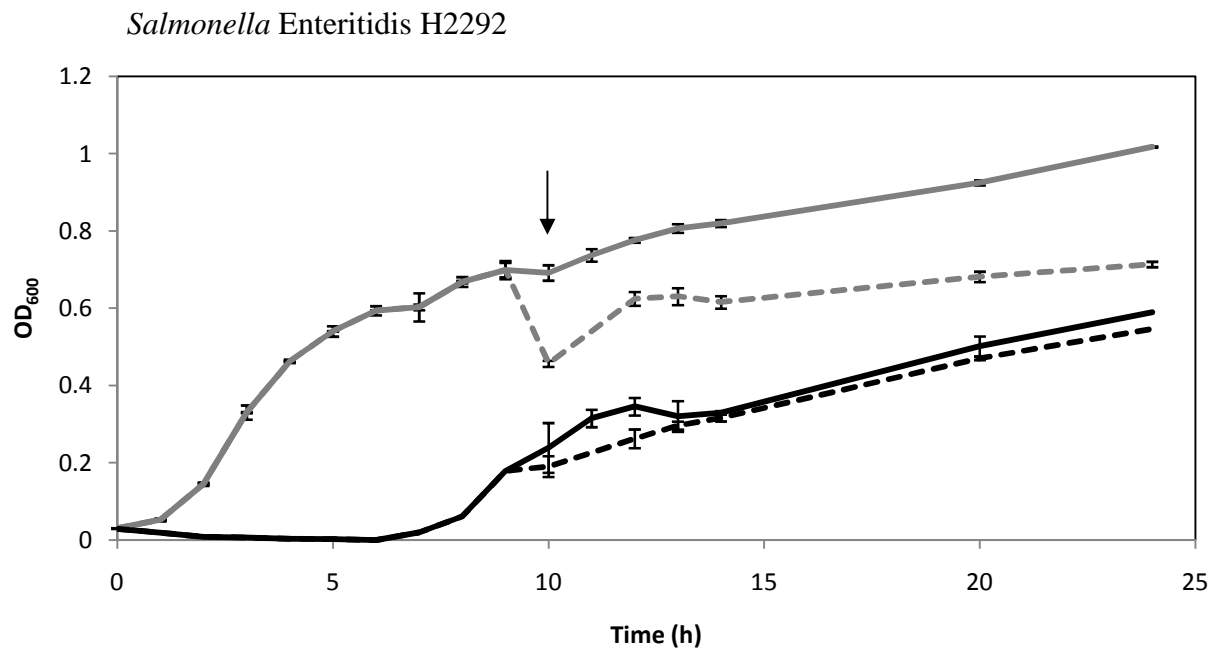


Figure 2.9c

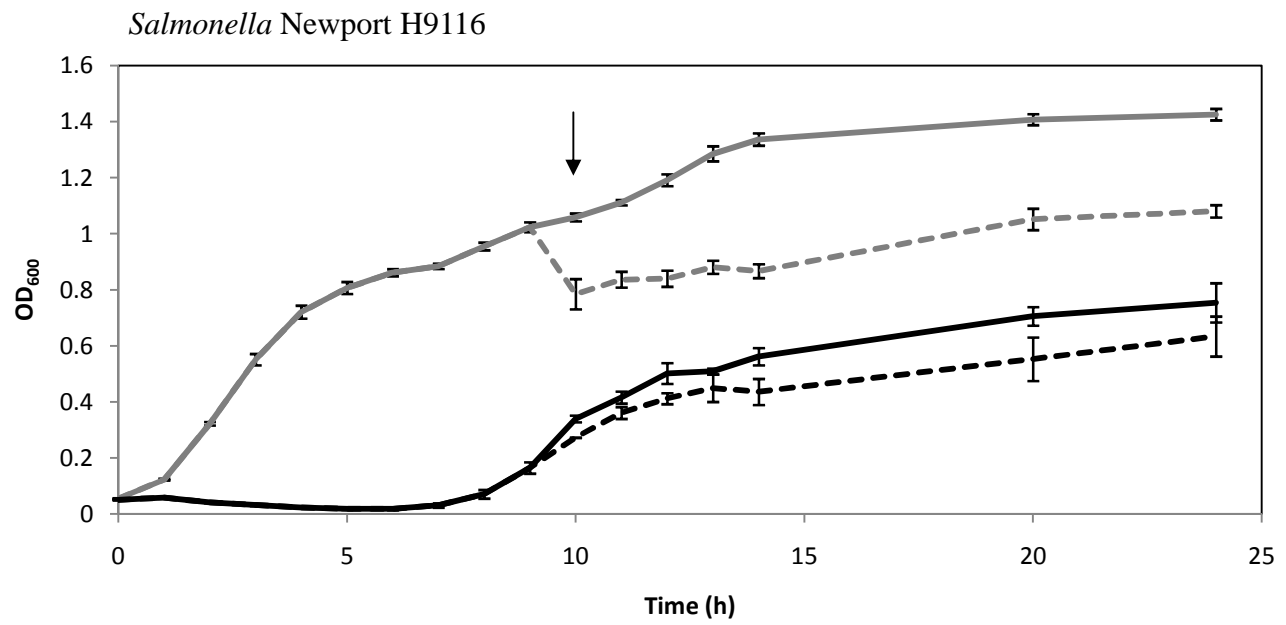


Figure 2.9d

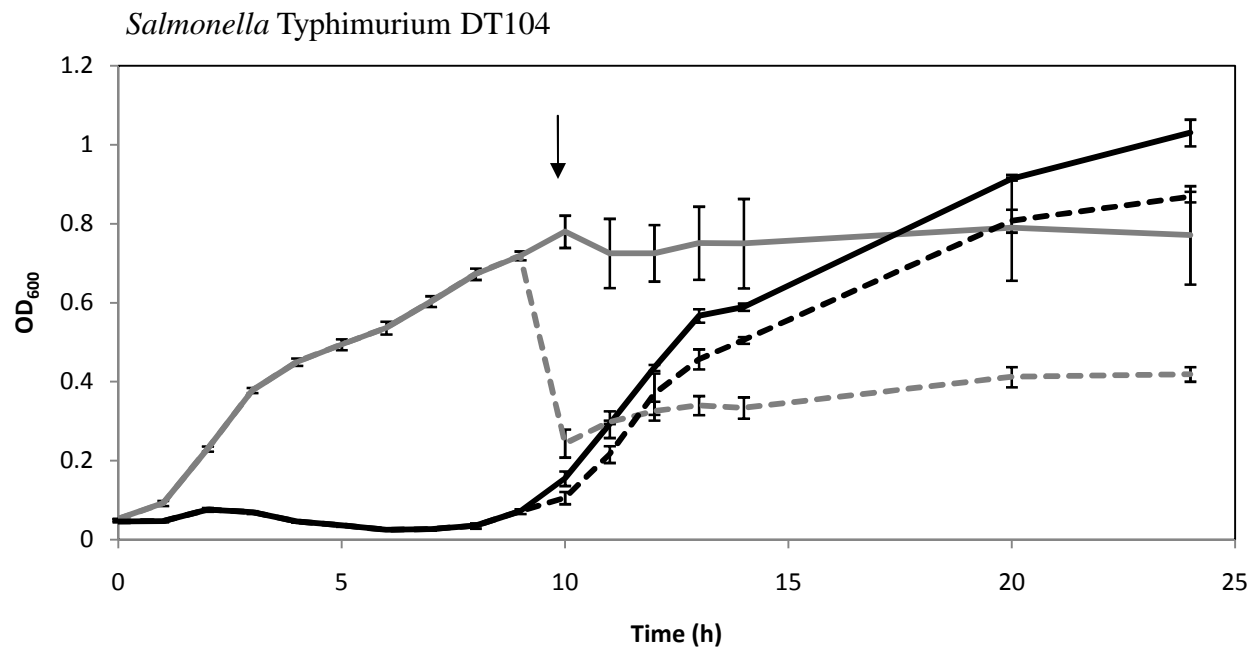
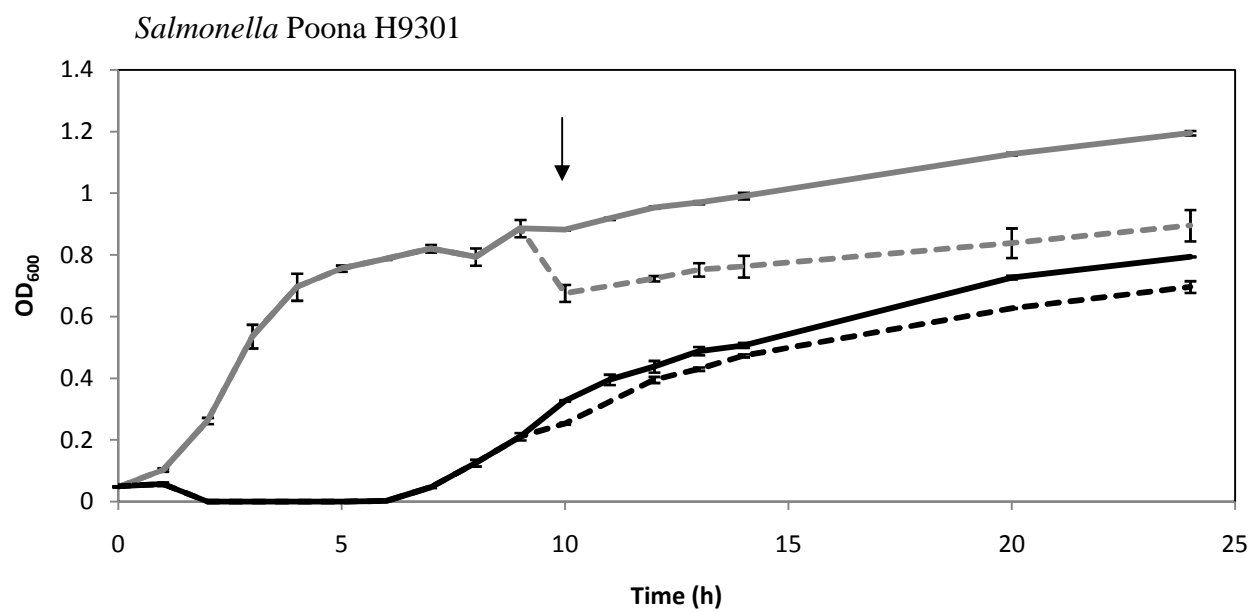


Figure 2.9e



CHAPTER THREE

APPLICATION OF PHAGE COCKTAILS TO COMPOST INOCULATED WITH
Escherichia coli O157:H7 OR *Salmonella* TYPHIMURIUM

Abstract

The ability of pathogenic bacteria to persist within the composting environment and the potential for agricultural contamination has led researchers to look for additional methods to treat waste materials. This study focused on the application of bacteriophage cocktails to both autoclaved and non-autoclaved dairy compost that had been inoculated with 10^5 CFU/g of either *Salmonella* Typhimurium or *Escherichia coli* O157:H7. Reductions of 2.34, 2.41 and 2.56 logs of *Salmonella* Typhimurium 8243 were observed in non-autoclaved compost at phage MOIs of 1, 10 and 50, respectively. An identical study using *E. coli* O157:H7 strain 0923-21 resulted in ca. 0.17, 1.21 and 1.5 log reductions of the pathogen in non-autoclaved compost.

Compost prepared at water activity levels of 0.9, 0.95 and 0.98 yielded no pathogen reductions as a result of phage treatment in any of the trials. Reductions of 2 to 3 logs of *Salmonella* were seen in non-autoclaved compost at a water activity near 1, whereas *E. coli* O157:H7 in non-autoclaved compost had a one log decrease after phage treatment. A slight reduction of 0.2 log₁₀ CFU/g of *Salmonella* was observed in the inoculated autoclaved compost with water activity near 1, whereas *E. coli* O157:H7 populations were reduced by about one log. Neither reduction resulted in bacterial

populations below the initial inoculation levels due to the initial pathogen re-growth prior to phage treatment.

The results indicate that bacteriophage cocktails are effective at reducing bacterial populations in compost under certain conditions such as an appropriate ratio of phage to bacteria and high water activity. Furthermore, the presence of background micro-organisms which prevent further growth of *E. coli* O157:H7 and *Salmonella* is necessary for significant reductions by phage.

Introduction

Composting is a complex process designed to eliminate the presence of harmful micro-organisms while producing a nutrient-rich substrate, suitable for land application (MØlbak et. al., 2005). When performed properly, organisms such as *Salmonella* and *Escherichia coli* O157:H7 are reduced to undetectable levels in most situations (Misra et. al., 2003). Some studies, however, have found that both *Salmonella* spp. and *E. coli* O157:H7 are able to survive if composting is performed improperly (Jones et. al., 1982). Furthermore, the surfaces of compost heaps have been shown to reach insufficient temperatures for the complete inactivation of pathogenic bacteria (Shepherd et. al., 2007).

A rise in the demand for organically grown fruits and vegetables has increased the need for safer crop amendments. In spite of the growing awareness of the potential dangers of pathogen presence in crops, multiple outbreaks of food-borne illness associated with fresh produce have recently occurred (Nygard et. al., 2004; FDA, 2006). The persistence of human pathogens such as *Salmonella* and *E. coli* O157:H7 in compost

has led researchers to explore different avenues for pathogen reduction such as irradiation or ammonia supplementation (Monteith et. al., 1986; Park and Diez-Gonzales, 2003). To date, no studies have explored the potential for bacteriophage application to control pathogens from compost.

Bacteriophages are viruses that are able to infect and destroy specific strains of bacteria. They have been successfully implemented for the reduction of pathogens in live animals (Atterbury et. al., 2007; Smith and Huggins, 1983; Sheng et. al., 2006), fresh produce (Leverentz et. al., 2003; Pao et. al., 2004), and meat products (Whichard et. al., 2003; Greer et. al., 1988). The objectives of this study were to utilize previously tested and characterized bacteriophage cocktails to reduce *Salmonella* or *E. coli* O157:H7 populations in compost, and to determine the necessary conditions for successful elimination of these pathogens.

Materials and Methods

Bacterial and phage stock preparation: Bacterial strains *Salmonella* Typhimurium 8243 and *E. coli* O157:H7 F06M-0932-21 (spinach outbreak strain from California Dept. of Health) were grown overnight in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) at 37°C with shaking. Cells were washed in 0.85% saline to an optical density at 600 nm wavelength of 0.5 (estimated at 10⁹ CFU/ml) and diluted 1:100 in saline prior to inoculation in compost. Phage stocks, prepared as described in Chapter 2, were warmed for 30 minutes at 37°C and diluted to desired concentration in SM buffer [100 mM NaCl, 8 mM MgSO₄*7H₂O, 50 mM Tris-Cl (pH 7.5)]. Phage titer

was determined by a double agar layer (DAL) plaque assay as described in Chapter 2. The cocktail for *Salmonella* trials was prepared for bacterial concentrations of 10^5 CFU/g at the following MOI as determined in Table 2.3: H4717 S5p2, 48.9; H2292{2}, 0.05; H9301 (1)-2, 0.5; FO, 0.5; ME-18 p2, 0.05. *E. coli* O157:H7 cocktail was prepared with equal MOIs of 12.5 for all phages: E0654, E01993, E009 and SPp. Cocktails were then further diluted to 1:5 or 1:50 for MOI study.

Compost preparation: Finished dairy manure compost (>60 days post composting) was taken from a field study from our lab and was composed of cow manure/sawdust-calf feces mixture, wasted feed, old hay and vegetable wastes (squash and plant residues) at a ratio of 10:2:2:2, respectively. The autoclaved compost was prepared by spreading 2,000 g of compost on plastic trays and autoclaving at 121°C for 20 minutes, consecutively for three days. Non-autoclaved compost (2,000 g) was dried at room temperature for 2 days to obtain water activity levels below 0.9. Sterile tap water was used to prepare 300 g of autoclaved and non-autoclaved composts at water activities of 0.9, 0.95, 0.98 and 0.999 measured with an AquaLab water activity meter (Decagon Devices, Pullman, WA). Moisture contents were determined using an IR-35 moisture analyzer (Denver Instruments, Denver, CO).

Phage challenge study with different MOIs: Three milliliters of *Salmonella* Typhimurium 8243 or *E. coli* O157:H7 at 10^7 CFU/ml were sprayed over the surface of 600 g of non-autoclaved finished dairy compost brought to a moisture content of 50%. Samples were aseptically mixed by hand for 5 mins on sterile trays covered in aluminum foil, wrapped and stored at room temperature overnight. The samples were divided into

four 150 g portions and sprayed with 3 ml of either SM buffer or phage cocktail at MOIs of 1, 10 and 50. At selected intervals, sampling was performed in duplicate by aseptically removing 10 g of compost with a tongue depressor and mixing with 90 ml of 0.85% saline in sterile sample bags. Bags were shaken vigorously by hand for 100 s and samples were diluted appropriately in 0.85% saline and spiral plated (Autoplate®4000, Bethesda, MD) onto either XLT-4 supplemented with rifampin (*Salmonella*) or TSA supplemented with rifampin at 100 µg/ml (*E. coli* O157:H7).

Phage treatment of compost with different water activities: Three milliliters of *Salmonella* Typhimurium 8243 or *E. coli* O157:H7 at 10^7 CFU/ml were sprayed over the surface of 300 g of autoclaved and non-autoclaved finished dairy compost at each water activity (0.9, 0.95, 0.98 and 0.999). Samples were aseptically mixed as described above and the following day sprayed with 3 ml of either phage cocktail or SM buffer (control). Both *Salmonella* and *E. coli* O157:H7 in phage treated composts were sampled and enumerated as described above. Background bacteria from the compost were counted by sampling as described above and spread-plating 100 µl of each dilution onto TSA with a sterile hockey stick.

Statistical analysis: Bacterial count data were converted to \log_{10} CFU/g for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design was conducted to determine if general differences existed between treatment means using the general linear model (GLM) procedure. Specific comparisons among different phage treatments were accomplished with Tukey's test. All statistical analysis was performed using the Statistical Analysis System 9.1 (SAS; SAS Institute, Cary, NC).

Results

Effect of multiple MOIs on compost treated with a bacteriophage cocktail

The results of using a bacteriophage cocktail at multiple MOIs against *E. coli* O157:H7 suggest that the ratio of phage to bacteria plays a key role in bacterial reduction (Fig. 3.1a). There was a slight reduction of 0.17 log effect when the MOI was 1, while a ten-fold increase in phage resulted in reductions of 1.21 logs ($p < 0.05$) in bacterial population. Similarly, a fifty-fold increase resulted in even higher ($p < 0.05$) reductions. In contrast to the *E. coli* O157:H7 results, the bacteriophage cocktail specific to *Salmonella* Typhimurium was highly ($p < 0.05$) effective at all ratios (Fig. 3.1b). However, statistical analysis of plate count data revealed no difference ($p > 0.05$) among MOIs of 1, 10 and 50 which resulted in reductions of 2.34, 2.37 and 2.56 logs, respectively.

Optimal composting water activity for phage infection

It was determined that the water activities of 0.9, 0.95, 0.98 and 0.999 in dairy compost used in this study had moisture contents of 14, 18, 23 and 50%, respectively. There were ca. 3 to 4 logs of bacterial reduction at water activities of 0.9 and 0.95, prior to phage treatment. During overnight acclimatization, both *E. coli* O157:H7 0923-21 and *Salmonella* Typhimurium 8243 had about 2 to 3 logs of growth for autoclaved compost at a water activity near 1, prior to phage treatment. Autoclaved compost at a water activity of 0.98 also had about one log increase for *E. coli* O157:H7. Neither bacteriophage cocktail had an effect on *Salmonella* Typhimurium or *E. coli* O157:H7 in either

autoclaved or non-autoclaved compost at water activities of 0.9 and 0.95 (Figs. 3.2 and 3.3).

The autoclaved compost used in this study contained a background microbial population of ca. 2 logs. At a water activity near 1, the reductions of *Salmonella* Typhimurium 8243 by phage treatment was ca. 0.3 log, yet pathogen populations remained almost 2 logs higher than the initial inoculum. *E. coli* O157:H7 in autoclaved compost with water activity near 1 was reduced by about one log, however pathogen levels were still higher than the initial inoculum. There was no significant ($p>0.05$) difference in pathogen populations between control and phage treatments in the autoclaved compost at a water activity of 0.98 (Table 3.1).

The non-autoclaved compost had significant reductions of both pathogens at water activity near 1. The phage cocktail reduced *Salmonella* Typhimurium by nearly 3 logs, whereas the *E. coli* O157:H7 levels were decreased by less than one log (Table 3.2). At a water activity of 0.98, phage treatment resulted in *Salmonella* Typhimurium reductions ($p<0.05$) after 24 h, as compared with no effect of phages on *E. coli* O157:H7.

Discussion

The recent demand for organic products has left few options for fertilizers and nutrient amendments outside of animal wastes. Gerba and Smith (1995) states that since waste materials can harbor as many as 150 enteric pathogens, treatment is essential prior to human contact, especially involving the food supply. Composting has already been utilized and shown to be effective at eliminating pathogenic bacteria (Misra et. al., 2003).

Furthermore guidelines have been proposed to ensure the composting process is effective. Richard et. al.(1998) suggest moisture contents of between 40 – 60% as well as Carbon-to-Nitrogen ratios of 20:1 – 40:1 for active composting. The results of both the MOI study and water activity study here highlight the fact that optimal phage effectiveness occurs in higher water activities. The compost surface has been identified as a possible source for the prolonged survival of pathogens (Shepherd et. al., 2007). Therefore, when the composting process is in the early phase, the possibility exists for bacteriophage use to supplement effective composting as a safeguard against not only pathogen persistence but also secondary contamination from wild animals or cross contamination from animal waste.

Bacteriophage application in autoclaved compost

In the absence of competition, with abundant nutrients, it is not surprising that a bacterial inoculum of 10^5 CFU/g would flourish in autoclaved compost (Table 3.1). Pietronave et. al. (2004) found that in sterile composts at moisture contents of 10, 40 and 80%, *Salmonella arizonae* grew from an initial inoculum of 10^5 CFU/g by 1 to 4 logs within 24 h. Similar results were found for *E. coli*, suggesting that in the absence of competitive microorganisms, bacteria can grow in compost as low as 10% moisture. A study by Soares et. al. (1995) however found that *E. coli* re-growth was inhibited at moisture contents below 30%. All of our samples with water activities except for 0.999 were at moisture contents below 30%. Our study found that re-growth did occur at water

activities of 0.98 (m.c. ~23%) and 0.999 (m.c. 50%), however reductions of 2 to 3 logs occurred in water activities of 0.9 (m.c. ~14%) and 0.95 (m.c. ~18%).

It has been demonstrated that as the phage to bacteria ratio (MOI) is increased in compost, the number of target bacteria recovered declines (Fig. 3.1a & b). This is potentially one reason why little to no effect was seen in autoclaved compost at a water activity near 1. Since the bacterial populations at time 0 ranged from 7 to 8 logs, the actual MOI was around 0.1. It would seem however, that larger numbers of host would be more susceptible to bacteriophage regardless of the ratio. This is especially true considering that actively growing cells are more likely to be infected by a bacteriophage. The possibility exists that even though water activity was high, the number of bacteria that were present in the compost were repopulated so well that phage movement in solid matrix was insufficient to result in large reductions.

There may also be unknown factors as a result of autoclaving the compost. Changes in the pH or structure of the compost itself may have contributed to the inactivation of some of the bacteriophages. A study by Vettori et. al. (1999) found that a bacteriophage infecting *Bacillus* would rapidly bind to certain types of clay particles in the environment. The effect that autoclaving has on certain components of the compost is unknown, however the materials themselves were observed to be much more brittle and fine when compared to non-autoclaved compost. The presence of looser particles as a result of autoclaving may have caused more phages to become permanently bound and inactive.

Overall, the lack of effect of bacteriophage treatment in autoclaved compost was unexpected. It would be interesting to investigate whether phage treatment at the exact time of inoculation would prevent the re-growth of either *Salmonella* or *E. coli* O157:H7. Further study involving higher moisture levels or a compost slurry may increase the effectiveness of a phage cocktail in autoclaved as well as non-autoclaved compost. A study by Lehman (2008) utilized a surrogate host, which was non-pathogenic, but allowed for the amplification of their test phage. This may provide a dual effect in compost by allowing for amplification of the bacteriophage as well as a direct competitive effect against the target bacterium.

Bacteriophage application in non-autoclaved compost

The use of a phage cocktail specific to *Salmonella* Typhimurium 8243 resulted in a greater than 99% reduction in viable bacteria within 24 h at a water activity of near 1. The fact that no reductions were seen in compost with about half the moisture ($a_w=0.98$) signifies the importance of fluid movement within the sample. It is fortunate that the optimal conditions for bacteriophage treatment also fall within the optimal conditions for proper composting in the early phase, in terms of moisture content.

The results from the trial with *E. coli* O157:H7 reveal pathogen reductions of less than one log, which is less than the reductions obtained during the optimization studies with MOI (Fig. 3.1a). Although unlikely, there is a possibility that the phage titer dropped between experiments. This could account for the lessened effectiveness since an MOI of 1 only caused 0.17 log reductions and an MOI of 10 was just over one log. It is

also possible that the addition of several more bacteriophages would be able to enhance the effects of the treatment. Chase (2006) used a cocktail containing 37 phages for *E. coli* O157:H7 and found that no strains were able to develop resistance. They reported the complete elimination of 4 logs of *E. coli* O157:H7 inoculated into milk within 72 h. When the phage cocktail treatment was performed on calves inoculated with 10^8 CFU of *E. coli* O157:H7, the effect was short-lived.

Conclusion

This study was a first step in the development of intervention techniques that will be employed in the future for effective treatment of pathogens in composts and manures. There is a great deal of work yet to be done, however the results are promising. We have demonstrated conclusively that bacteriophage can reduce *Salmonella* and *E. coli* O157:H7 in compost. Some of the limitations of phage application have also been discovered in their lack of effectiveness under low water activity conditions. Currently, phage treatment of compost would be insufficient alone, however potentially successful in conjunction with the composting process. Reductions of a few logs may be enough to impact the effectiveness of downstream applications in the food production chain.

Acknowledgements

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Figure Legend

Figure 3.1a-b: The use of phage cocktails for the elimination of *E. coli* O157:H7 0923-21 (a) and *Salmonella* Typhimurium 8243 (b) in finished dairy compost at MOIs of 1, 10 and 50. Means with the same letter at each sampling date are not significantly different ($P>0.05$).

Figure 3.2a-b: *E. coli* O157:H7 detection in autoclaved and non-autoclaved compost at $a_w=0.9$ (a) $a_w=0.95$ (b) after phage treatment. Means with the same letter at each sampling date are not significantly different ($P>0.05$).

Figure 3.3a-b: *Salmonella* Typhimurium detection in autoclaved and non-autoclaved compost at $a_w=0.9$ (a) $a_w=0.95$ (b) after phage treatment. Means with the same letter at each sampling date are not significantly different ($P>0.05$).

Table 3.1 Detection of *Salmonella* Typhimurium 8423 and *E. coli* O157:H7 0923-21 in autoclaved compost after phage treatment

Bacteria	Sample Time (h)	Water Activity			
		0.98		0.999	
		Control	Treatment	Control	Treatment
<i>E. coli</i> O157:H7	0	6.49 ± 0.03 A ^a	6.49 ± 0.03 A	7.43 ± 0.05 A	7.43 ± 0.05 A
	4	6.34 ± 0.03 A	6.45 ± 0.06 A	7.66 ± 0.08 A	6.54 ± 0.05 B
	8	6.20 ± 0.18 A	6.19 ± 0.04 A	7.54 ± 0.03 A	6.34 ± 0.11 B
	24	6.13 ± 0.06 A	6.10 ± 0.08 A	7.43 ± 0.09 A	6.56 ± 0.03 B
<i>Salmonella</i> Typhimurium	0	5.49 ± 0.04 A	5.49 ± 0.04 A	7.48 ± 0.03 A	7.48 ± 0.03 A
	4	5.39 ± 0.05 A	5.43 ± 0.09 A	7.57 ± 0.05 A	7.31 ± 0.09 A
	8	5.12 ± 0.06 A	5.15 ± 0.04 A	7.58 ± 0.08 A	7.32 ± 0.14 A
	24	5.00 ± 0.06 A	4.87 ± 0.11 A	7.42 ± 0.05 A	7.13 ± 0.08 A

^aValues with different capitalized letters are statistically different (p<0.05) for the compost with the same water activity at each sampling time for individual strains.

Table 3.2 Detection of *Salmonella* Typhimurium 8423 and *E. coli* O157:H7 0923-21 in non-autoclaved compost after phage treatment

Bacteria	Sample Time (h)	Water Activity			
		0.98		0.999	
		Control	Treatment	Control	Treatment
<i>E. coli</i> O157:H7	0	5.05 ± 0.04 A ^a	5.05 ± 0.04 A	5.99 ± 0.19 A	5.99 ± 0.19 A
	4	5.01 ± 0.04 A	4.97 ± 0.13 A	6.14 ± 0.18 A	5.33 ± 0.04 B
	8	4.83 ± 0.06 A	4.81 ± 0.14 A	5.91 ± 0.05 A	5.21 ± 0.06 B
	24	< 4.30	< 4.30	5.71 ± 0.05 A	5.16 ± 0.18 B
<i>Salmonella</i> Typhimurium	0	5.49 ± 0.04 A	5.49 ± 0.04 A	5.51 ± 0.08 A	5.51 ± 0.08 A
	4	5.47 ± 0.13 A	5.25 ± 0.18 A	5.28 ± 0.05 A	2.78 ± 0.00 B
	8	5.18 ± 0.04 A	5.28 ± 0.05 A	5.29 ± 0.03 A	2.53 ± 0.15 B
	24	5.28 ± 0.04 A	5.10 ± 0.02 B	5.04 ± 0.14 A	2.38 ± 0.15 B

^aValues with different capitalized letters are statistically different (p<0.05) for the compost with the same water activity at each sampling time for individual strains.

Figure 3.1a

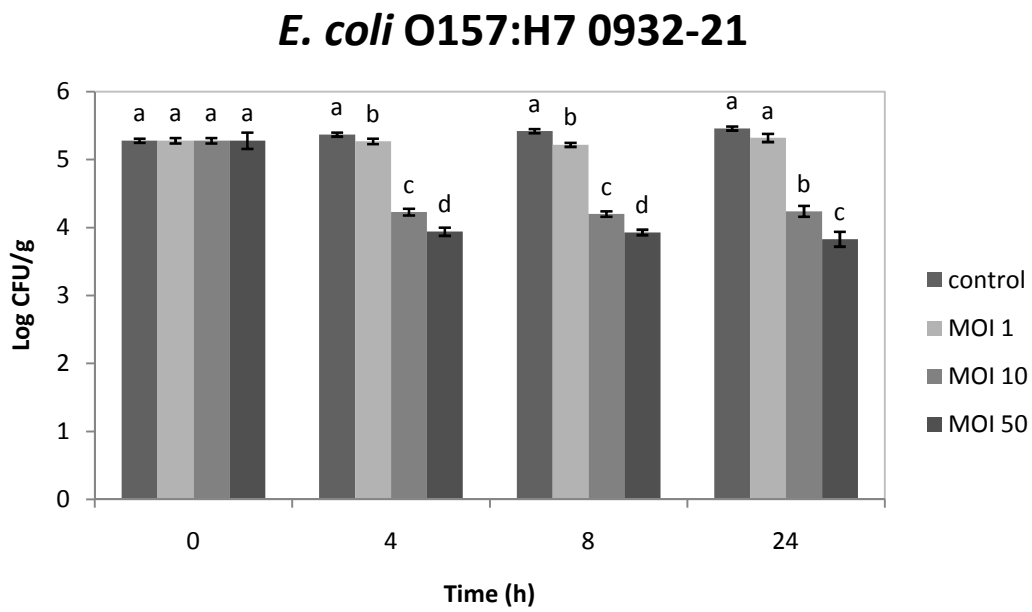


Figure 3.1b

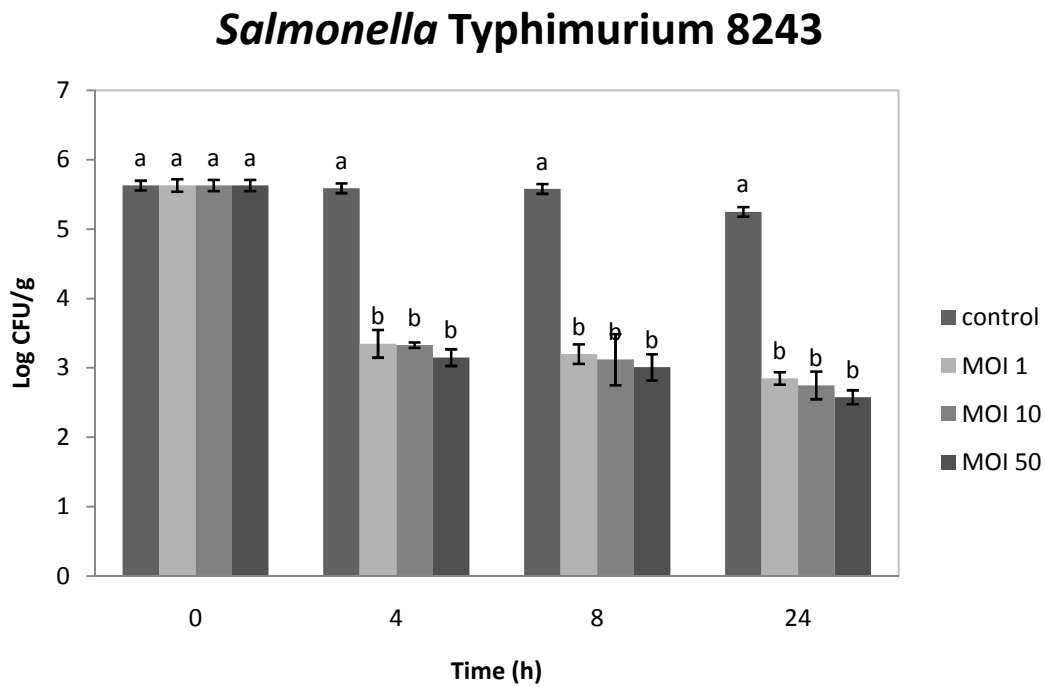


Figure 3.2a

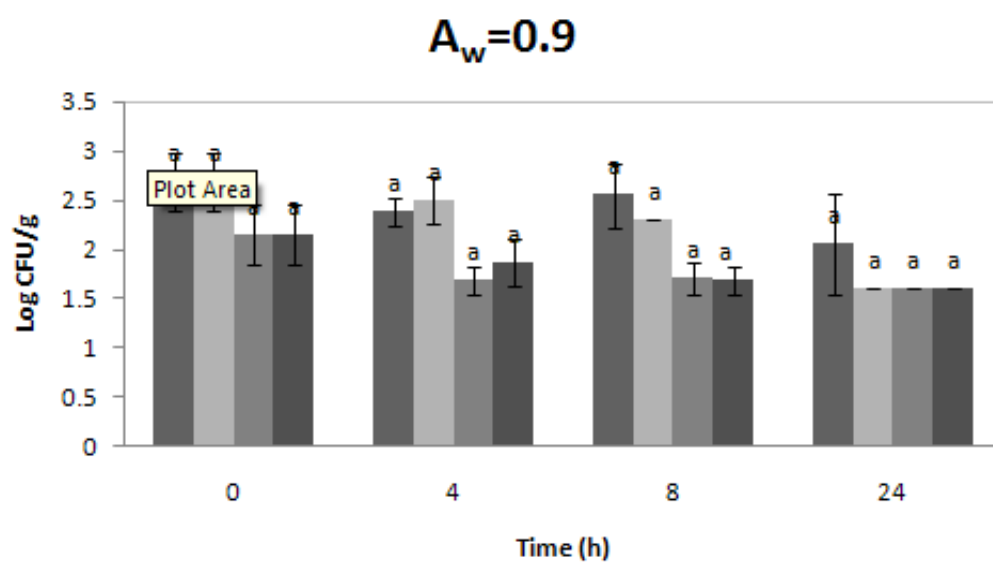


Figure 3.2b

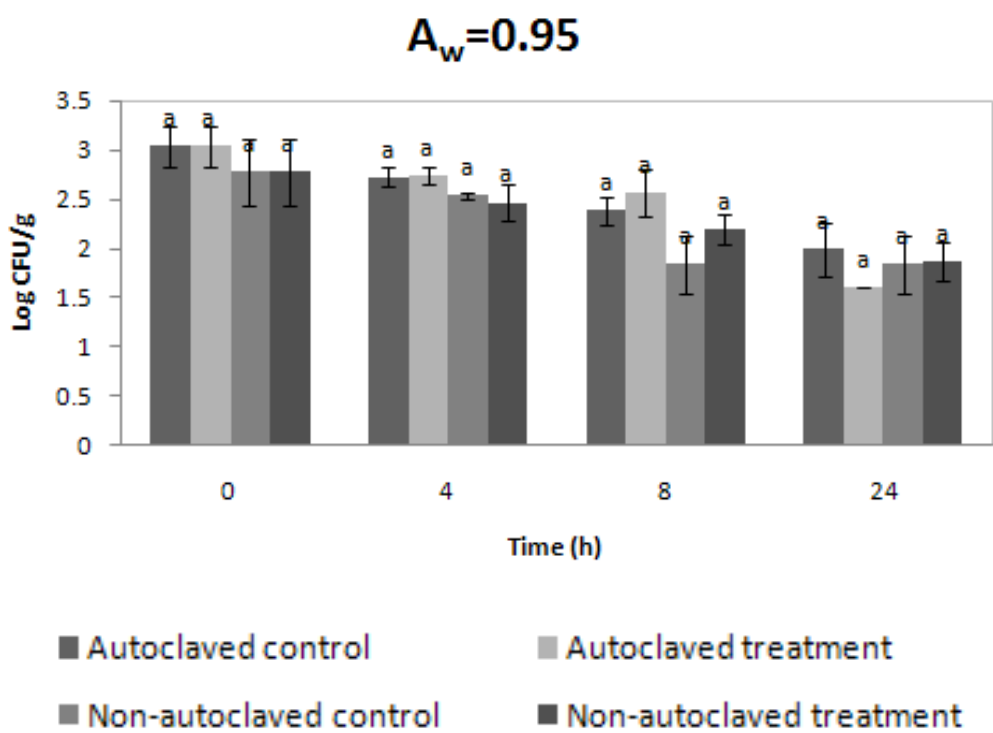


Figure 3.3a

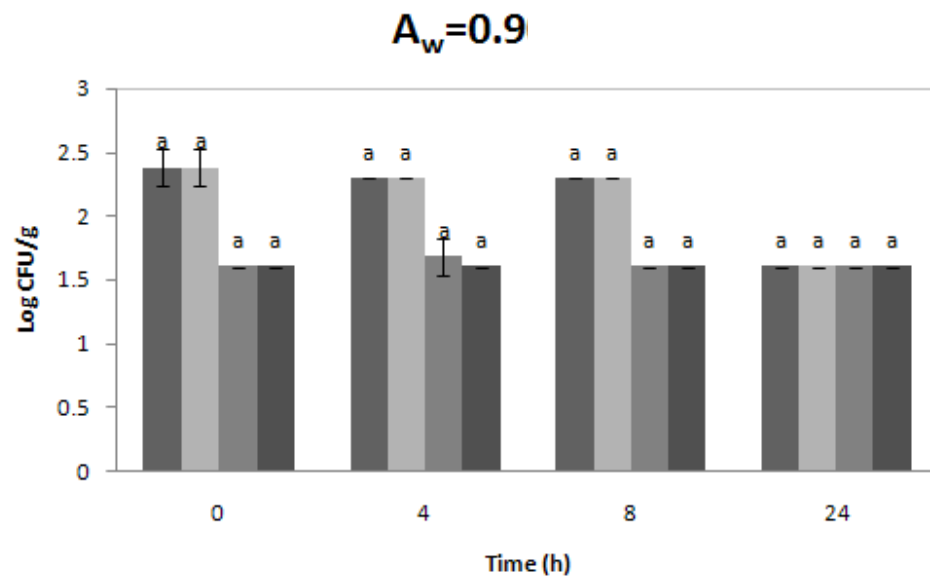
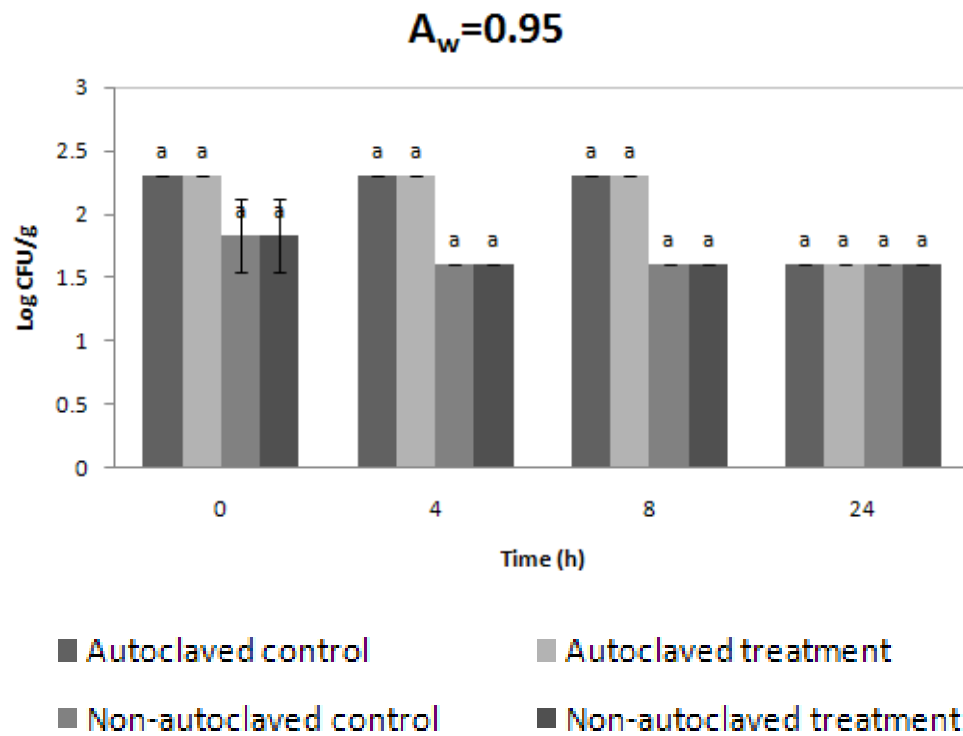


Figure 3.3b



CONCLUSION

Bacteriophage therapy is becoming more and more popular because it is relatively inexpensive, harmless to humans and animals, and there is literally a phage for every strain of bacteria in existence. In this study, we isolated and characterized bacteriophages specific to *Salmonella* spp. and *E. coli* O157:H7 using standard enrichment procedures. We were able to optimize and develop reliable methods for the characterization of bacteriophages, by restriction digests, electron microscopy and standard plating. Previous research on the methods of characterizing and utilizing bacteriophages allowed us to develop optimal phage cocktails for use in a novel way; inoculation directly into compost for the elimination of pathogenic bacteria. The initial results of this study are promising in that large reductions were observed for both *E. coli* O157:H7 and *Salmonella* Typhimurium with the use of phage cocktails. Further study will reveal that the potentially unlimited abundance of phages in nature allow for even more effective phage cocktails to be developed. To our knowledge this is the first study to test the ability for phage cocktails to reduce pathogen presence in compost.